Potassium Homeostasis and Salinity Tolerance in Barley: Physiological and Genetic Aspects

by

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

Zhonghua Chen

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List of Abbreviations

$^1O_2$	singlet oxygen
ABA	abscisic acid
ADP	adenosine diphosphate
a_K	potassium activity
AKT/KAT	*Arabidopsis* inward K^+ channel
ANOVA	analysis of variance
ANRA	Australian Natural Resources Atlas
APX	ascorbate peroxidase
ATP	adenosine triphosphate
ATPase	enzymes catalyse the decomposition of ATP into ADP and Pi
BADH	betaine aldehyde dehydrogenase
BC_1 / BC_2	backcross generation one / two
C_3 / C_4	C_3 / C_4carbon fixation
CAT	catalase
cDNA	complementary deoxyribonucleic acid
CHX	cation/hydrogen exchanger
C_i	intercellular CO_2 concentration
CMO	choline monooxygenase
CO_2	carbon dioxide
CPA	cation proton antiporter
D	genetically additive component of variation
DAPC	depolarisation activated K^+ channel
df	degree of freedom
DTT	dithiothreitol
DW	dry weight
EDTA	ethylene diamine tetracetic acid
EGTA	ethylene glycol tetracetic acid
E_K	K^+ equilibrium potential
E_m	plasma membrane potential
EST	expressed sequence tag

FAO	Food and Agriculture Organisation of the United Nations
F_v/F_m	maximal quantum efficiency of PSII
FW	fresh weight
GORK	guard cell K^+ outward-rectifier
GPX	glutathione peroxidase
GR	glutathione reductase
g_s	stomatal conductance
H	genetically dominance component of variation
h^2_B	broad sense heritability
h^2_N	narrow sense heritability
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKT	high-affinity K^+ transporter
HPLC	high performance liquid chromatography
HSHS	half-strength Hoagland's solution
HVP	vacuolar H^+-inorganic pyrophosphatase
IBDU	isobutylenediurea
IMT	*myo*-inositol methyl transferase
KCO	Ca^{2+} activated K^+ outward rectifier
KIRC	K^+ inwardly-rectifying channel
KORC	K^+ outwardly-rectifying channel
KUP/HAK/KT	K^+ uptake permease/high-affinity K^+ transporter/K^+ transporter
LCT	low-affinity cation transporter
LIX	liquid ion exchanger
M6PR	mannose-6-phosphate reductase
MES	2-(*N*-morpholino) ethanesulfonic acid
MIFE	microelectrode ion flux measurement
MOPS	3-(*N*-morpholino) propanesulfonic acid
MS	mean of squares
NAD^+	nicotinamide adenine dinucleotide
NHX	sodium/hydrogen antiporter
NORC	non-selective outward rectifying cation channel
NPQ	nonphotochemical quenching
NSCC	non selective cation channel

$O_2^{\cdot}$	superoxide
$OH^{\cdot}$	hydroxyl radicals
P5C	Δ-1-pyrroline-5-carboxylate
P5CR	Δ-1-pyrroline-5-carboxylate reductase
P5CS	Δ-1-pyrroline-5-carboxylate synthetase
PCR	polymerase chain reaction
PDA	photodiode array
PEG	polyethylene glycol
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
P_n	net CO_2 assimilation
POD	peroxidase
ProT	proline transporter
PSI / PSII	photosystem I / II
PVC	polyvinyl chloride
PVP	polyvinylpyrrolidone
QACs	quaternary ammonium compounds
qP	photochemical quenching
QTL	quantitative trait locus
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
RWC	relative water content
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SKOR	stellar K^+ outward-rectifier
SOD	superoxide dismutase
SOS	salt overly sensitive
SS	sum of squares
SV / FV	slow / fast-activated vacuolar channel
SWC	shoot water content
T-DNA	transferred deoxyribonucleic acid
TEA^+	tetraethylammonium chloride
Tris	trishydroxymethylaminomethane
tRNA	transfer ribonucleic acid
TSW	thousand seed weight

V-ATPase	vacuolar type H^+-ATPase
VHA	the catalytic subunit of the vacuolar H^+-ATPase
VIC	voltage independent channel
VPPase	vacuolar pyrophosphatase
Wr / Vr	covariance / variance
WT	wild type
Φ_{PSII}	quantum yield of photosynthetic electron transport

Abstract

Developing salt-tolerant crops is central to remediation of salinity affected land and to meet increasing global food demand. Salt tolerance is a polygenic trait involving multiple mechanisms, with contributions from genetic, developmental, physiological and environmental interactions. The molecular and physiological mechanisms of plant salt tolerance have been intensively investigated with most of the studies focused on detrimental effects of Na^+. Accordingly, most of the plant breeding for salt tolerance has been focused almost exclusively on excluding Na^+ from uptake and/or transport to shoot. Salt-induced perturbations in K^+ homeostasis are often recognised as being of secondary importance or even ignored by many researchers. This work argues that surmounting those constraints may open new avenues for breeding for salt-tolerant crops. Various plant physiological and genetic techniques were employed to test the hypothesis that the K^+ retention in barley under saline conditions is central to maintain the cytosolic K^+/Na^+ ratio and hence that salt-induced loss of K^+ from seedling roots can be used for effective selection and breeding of salt-tolerant barley.

A comprehensive study was undertaken comparing whole-plant and cellular responses to salinity. Using seven barley cultivars contrasting in salt tolerance, a strong negative correlation was observed between the magnitude of K^+ release from the roots of young seedlings and salt tolerance of mature plants judged by various physiological indices under saline conditions in glasshouse experiments. This suggested that K^+ loss from the mature zone of intact 3-d old roots following 1 h pre-treatment with 80 mM NaCl can be used as a reliable screening indicator for salt tolerance in barley. A faster and more cost-effective procedure, based on the amount of K^+ lost from plant roots during exposure to NaCl, was developed for rapid screening of large numbers of seedlings.

To confirm the suitability of K^+ efflux trait as a screening method for breeding programmes, nearly 70 randomly selected barley cultivars were employed in glasshouse trials over two consecutive years to evaluate their responses to salinity. K^+ loss under salt stress was measured from roots according to the above method. NaCl-induced K^+ loss was found highly inversely correlated with relative grain yield, shoot biomass, plant height, net CO_2 assimilation, survival rate, and thousand seed

weight measured in the glasshouse experiments. Altogether, 60 out of 69 genotypes showed strong negative correlation ($r^2 > 0.6$) between the magnitude of K^+ loss from roots of young seedlings and plant salt tolerance. Further analysis showed that a few remaining cultivars that did not follow the above trend, showed a superior ability to prevent Na^+ accumulation in plant leaves and, thus, to maintain a higher cytosolic K^+/Na^+ ratio despite the K^+ loss.

A half diallel cross was made among six barley cultivars contrasting in salt tolerance for further understanding of the genetic behaviour of this trait. The variance (Vr) and covariance (Wr) analysis showed the existence of epistatic effects, which was confirmed by further tests using six different populations (parents, F_1, F_2, BC_1 and BC_2) from two different crosses. However, the tolerance was mainly controlled by additive effects with relatively smaller contributions from dominant and epistatic effects. A high heritability for salt tolerance based on salt-induced root K^+ loss in barley was found, thereby supporting the use of this technique in breeding programmes. Given the fact that root K^+ flux might be affected by a large number of other factors, the most reliable results are likely to be obtained while screening for salt tolerance of different genotypes or a doubled haploid population instead of segregating populations (e.g. when net ion fluxes are averaged between several samples).

Electrophysiological and biochemical techniques were also employed to investigate specific cellular mechanisms contributing to barley salt tolerance. It was found that, in salt-tolerant genotypes, multiple mechanisms are combined effectively in order to withstand saline conditions. These mechanisms include better control of membrane voltage, intrinsically higher H^+ pump activity, greater ability of root cells to pump Na^+ from the cytosol to the external medium or into the vacuoles, and higher sensitivity to supplemental Ca^{2+}. Meanwhile, no significant difference was found between salt-tolerant and -sensitive cultivars in their unidirectional $^{22}Na^+$ influx or in the density and voltage dependence of depolarisation-activated outward-rectifying K^+ channels (KORCs).

The impact of hydrogen peroxide (H_2O_2) (one of components of salt stress) on K^+ flux and the mitigating effects of glycine betaine and proline on NaCl-induced K^+ loss were found to be significantly higher in salt-sensitive barley genotypes. Higher accumulation of leaf glycine betaine, proline, and total amino acid concentration was found in salt-sensitive cultivars under salinity stress. Significant negative correlations

were observed between NaCl-induced K^+ loss and leaf glycine betaine and proline concentration. Potassium was the main contributor to cytoplasmic osmolality in salt-tolerant genotypes, while in salt-sensitive ones glycine betaine and proline contributed substantially to cell osmolality, compensating for reduced cytoplasmic K^+.

In conclusion, I propose that (1) the ability to maintain high cytosolic K^+/Na^+ ratio is the key feature for salt tolerance in barley; (2) multiple mechanisms and pathways control the high cytosolic K^+/Na^+ ratio in salt-tolerant barley, K^+ retention appears to be central to this process; (3) hyperaccumulation of known compatible solutes in barley does not appear to play a major role in barley salt tolerance; (4) K^+ efflux trait is highly inheritable; and (5) NaCl-induced K^+ efflux can be used as a reliable and cost-effective early screening indicator for salt tolerance in addition to other known indices. Breeding for salt tolerance should therefore be achieved by targeting K^+ homeostasis.

Chapter 1. Introduction

1.1. Salinity: a global threat to agricultural productivity

The damaging effects of salinity on agriculture have dramatically influenced ancient and modern civilisations (Jacobsen and Adams 1958; Jacobsen 1982). Soil salinity represents one of the most severe environmental problems in many parts of the world, including Australia. It is estimated that about 20% of cultivated lands and 33% of irrigated agricultural land worldwide is affected by high salinity, comprising nearly 7% of the world's total land area (Ghassemi et al. 1995; Yeo 1999; Munns 2005). Low precipitation, high evaporation, irrigation with saline water, and poor agricultural practice are among the major contributors to the increased soil salinity (Foolad 2004). In Australia, almost 820,000 ha of salinity-affected agricultural land are not suitable for production in the most affected regions such as southern Western Australia and the Murray-Darling basin. Furthermore, it is estimated that 17 million ha, a third of Australia's agricultural land, will be significantly affected by salinity by 2050 (ANRA 2001). In the Murray-Darling basin alone, the annual cost of salinity to agricultural production has been estimated at $260 million (Haw et al. 2000). Given food production needs to be increased to meet the growing demand from an increasing population (ca 50% by 2050; United Nation 2006 World Population Revision), developing salt-tolerant crops will be critical for agricultural sustainability (Flowers 2004; Cuartero et al. 2006).

1.2. Approaches to remediate soil salinity and enhance plant salt tolerance

To a certain extent, the salinity problem can be handled by reclaiming already affected areas by various engineering schemes for reclamation, such as drainage and irrigation with high-quality water. However, a major hurdle to this is its enormously high cost. Another approach is the exploitation or development of halophytes to accumulate high levels of salt, thus depleting soil salinity and remediating agricultural land (Epstein et al. 1980; Foolad 2004). Nonetheless, such remediation processes have had limited applicability. Thus plant breeding for salt tolerance remains high on agenda.

Six possible strategies have been proposed for developing salt-tolerant crops: (1) use of conventional breeding and selection; (2) developing interspecific hybrids of current crops; (3) using the variation existing in crops and their wild progenitors; (4) generating variation within existing crops by using recurrent selection, mutagenesis or tissue culture; (5) transformation via metabolic engineering; (6) identifying, cloning, and manipulating genes for salinity tolerance using molecular biology techniques (Flowers and Yeo 1995; Bohnert and Jensen 1996; Shannon 1997). Those strategies have been or can be applied to research work on barley salinity tolerance.

1.3. Barley and research on salt tolerance

Barley (*Hordeum vulgare* L.) is a major world crop ranked the fourth most important cereal after rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L. and *Triticum turgidum* L.), and maize (*Zea mays* L.) (Bengtsson 1992). Barley is grown in a wide range of environments with major areas of production in Asia, Europe, North America and Oceania (Harlan 1995). The annual world barley production is about 138 million tons with the top ten producing countries (in million tons): Russia (16.7), Canada (12.1), Germany (11.7), France (10.4), Ukraine (9.3), Turkey (9.0), Australia (6.6), United Kingdom (5.5), United States (4.6), and Spain (4.4) (FAO 2005).

There are several advantages in using barley to study genetic and physiological mechanisms for salinity tolerance: (1) it is a diploid species with a small number of chromosomes and a relatively short life cycle; (2) it has a large genetic, physiological and morphological variation; (3) it has well defined genetic maps based on morphological, cytological, protein and molecular markers; (4) as one of the most salt-tolerant crop species, barley has advantages over *Arabidopsis*: such as broader genetic diversity, higher salinity tolerance, easier to compare with other cereal crops, and more direct benefits to agriculture (Forster et al. 1997; Koornneef et al. 1997).

Varietal differences in barley yield under saline conditions have been shown in both glasshouse (Greenway 1962; Rawson et al. 1988) and field experiments (Richards et al. 1987; Slavich et al. 1990). Leaves of some barley varieties can hold Na^+ concentrations in excess of 500 mM without showing sign of injury (Greenway 1962; Rawson et al. 1988). Due to its genetic diversity, salt-tolerant barley contains genes closely related to salt tolerance genes from sea barleygrass (*Hordeum marinum*).

Barley is recognised as one of the most salt-tolerant crop species (Maas and Hoffman 1977), but salt-induced yield losses are very high because most of the elite barley genotypes have been deliberately selected and bred for high yield, malt and feed quality (Grausgruber et al. 2002).

However, there are still many unanswered questions on the physiological and molecular mechanisms that control barley salt tolerance. What are the vital mechanisms for barley salt tolerance? Why is screening barley for salt tolerance so difficult? What roles does K^+ play in response to high salinity? What are the major ion transporters that regulate K^+/Na^+ homeostasis? Those questions are the basis of my thesis.

1.4. Outline of the chapters

Chapter 1 is a general introduction of the impact of salinity on agriculture, remediations to soil salinity and improvement to plant salt tolerance, the advantages of using barley for studying salt tolerance, and the overall objectives of this thesis.

Chapter 2 is a literature review on the topic of physiological, genetic, and molecular mechanisms of plant salt tolerance, and the practical aspects of screening plants for salt tolerance. It also highlights plant Na^+ and K^+ transport.

Chapter 3 is a description of all of the general materials and methods which have been employed in this study.

Chapter 4 investigates applicability of the microelectrode ion flux measurement (MIFE) as a potential screening tool for barley salt tolerance and investigate ionic mechanisms underlying salinity tolerance in barley.

Chapter 5 focuses on screening barley genotypes for salt tolerance by using various physiological and agronomic indicators. It also discusses the correlation between those parameters and NaCl-induced K^+ efflux.

Chapter 6 tests the genetic evidence of K^+ efflux as an indicator of barley salt tolerance with different genetic models and its potential in assisting selection and breeding for salt-tolerant barley.

Chapter 7 investigates cellular mechanisms confirming salinity tolerance in barley roots and demonstrates that multiple mechanisms (e.g. higher PM H^+-ATPase activity, less PM depolarisation, and better ability in retaining K^+ and excluding Na^+)

are well-combined in order to withstand highly saline conditions.

Chapter 8 investigates the potential role of accumulation of compatible solutes in barley salt tolerance and mitigation of ROS stress.

Chapter 9 summarises major findings of previous chapters and concludes in the context of the overall objectives.

1.5. Aims of the research

The aim of this research was three-fold:

(1) to elucidate the underlying physiological and genetic mechanisms of salinity tolerance in different barley genotypes;

(2) to investigate the candidate ion channels and transporters relevant to salinity tolerance in barley using both MIFE and patch-clamp techniques;

(3) to evaluate potential screening methods for salinity tolerance in barley and to test the theoretical and practical aspects of using MIFE flux measurement for this purpose.

Chapter 2. Literature Review

2.1. Physiological mechanisms of plant salt tolerance

Salinity affects plant growth through osmotic and ionic effects (Bartels and Sunkar 2005; Yamaguchi and Blumwald 2005). Plants employ various strategies in response to salinity including (1) minimising initial Na^+ entry and Na^+ loading to the xylem; (2) intracellular compartmentation or allocation to old leaves (3) maintaining a relatively high cytosolic K^+/Na^+ ratio; (4) accumulating/synthesising optimal amounts of compatible solutes; and (5) increasing enzymatic and non-enzymatic antioxidant defence systems (Bohnert et al. 1995; Hasegawa et al. 2000; Tester and Davenport 2003).

2.1.1. Physiological response to salt stress

2.1.1.1. Germination

High salt concentrations in the seed-planting layer of soil frequently lead to germination failure (Fowler 1991), although as one of the most salt-tolerant crop species, some barley genotypes are reported to be able to germinate in seawater (i.e. 47 dS m^{-1}) (Mano and Takeda 1995, 1997a). Salinity affects germination by multiple mechanisms including prevention of water uptake and imposition of ionic stress (Bewely and Black 1982; Poljakoff-Mayber et al. 1994), reduction in hydrolysis and enzyme activities (Filho and Sodek 1988; Guerrier 1988), disturbance to nitrogen metabolism (Yapsanis et al. 1994; Dell'Aquila and Spada 1993), and imbalance of plant growth regulators (Khan and Rizvi 1994).

2.1.1.2. Plant growth and water status

Salinity in the soil reduces plant water uptake, leading to slower growth (Munns et al. 2006) along with a clear plant stunting as salt concentration increases (Wang and Nil 2000). The components of this effect on plant growth are the reduction in plant height, fresh and dry weight of leaves, stems, and roots, lower yield and deterioration of the quality of the product (Kumar 1995; AliDinar et al. 1999; Chartzoulakis and Klapaki

2000). One of the major causes for this growth reduction is inadequate photosynthesis owing to stomatal closure, inhibition of photosynthetic enzymes and limited CO_2 uptake (Zhu 2001b; Munns 2002).

Plant water status changes rapidly and relative water content (RWC) is a convenient method of assessing plant water status. The turgor pressure was unaffected by salinity, but the RWC was significantly decreased in some wheat genotypes that accumulated high levels of leaf Na^+. At the same time, the low leaf Na^+ accumulating genotypes significantly reduced both turgor pressure and RWC after 5 d of salt treatment (James et al. 2002; Rivelli et al. 2002), suggesting a rather complex relationship between plant water status and ionic relations under saline conditions.

2.1.1.3. Photosynthetic characteristics

Suppression of the photosynthetic capacity of many plant species by salt stress has been reported (Robinson et al. 1983; Ball and Farquhar 1984; Bowman and Strain 1987; Mäkelä et al. 1999). Decreased photosynthetic rate of salt-stressed plants is due to several factors: (1) reduced permeability to CO_2 due to dehydration of cell membranes; (2) reduction of CO_2 supply because of reduced stomatal conductance; (3) enhanced leaf senescence; (4) changes in enzyme activity and photochemical capacity; and (5) depression in metabolic processes by inhibition of CO_2 uptake (Seemann and Critchley 1985; Iyengar and Reddy 1996; Dubey 1997).

The relative importance of stomatal *vs* non-stomatal components of inhibition of photosynthesis under saline conditions has been a matter of controversy (Farquhar and Sharkey 1982; Flexas et al. 2004). It was found that mild to severe salt stress affects photosynthesis of C_3 plants through a decrease of stomatal and mesophyll conductance (Flexas et al. 2004). The low stomatal and mesophyll conductances were also found to be the main limitations on photosynthesis in salt-stressed cotton (*Gossypium hirsutum* L.), bean (*Phaseolus vulgaris* L.), olive (*Olea europaea* L.), as well as barley (Brugnoli and Lauteri 1991; Loreto et al. 2003; Jiang et al. 2006). However, Rivelli et al. (2002) reported that non-stomatal limitation had a greater effect on salt-treated sunflower (*Helianthus annuus* L.).

Due to the reduced photosynthetic activity, plants growing under saline conditions, particularly when exposed to excessive light often experience oxidative stress. This causes damage to photosystem II (PSII); although photosystem I (PSI)

remains essentially unaffected (Apel and Hirt 2004). It has been reported that the photosynthetic capacity of chloroplasts is depressed because salinity leads to the disintegration of the fine structure of chloroplasts, instability of the pigment protein complexes, destruction of chlorophyll, and changes in the quantity and composition of carotenoids (Dubey 1997; Fedina et al. 2002). However, the use of photosynthetic characteristics in screening plants for salt tolerance is complicated due to a lack of understanding of the specific mechanisms by which salt stress inhibits photosynthesis.

2.1.2. Sodium and potassium homeostasis

2.1.2.1. Sodium

For most plants, Na^+ is not an essential nutrient, although low Na^+ concentrations often stimulates plant growth in many species. To a large extent this is attributed to the role of Na^+ as an osmoticum in the vacuole, reducing the need for K^+ (Marschner 1995). Sodium has been shown to be essential for maximal growth in certain halophytic C_4 plant species, such as bladder saltbush (*Atriplex vesicaria*), fire bush (*Kochia childsii*), proso millet (*Panicum miliaceum* L.), and saltgrass (*Distichlis spicata* L.) (Brownell and Crossland 1972; Flowers et al. 1977; Subbarao et al. 2003). In C_4 species, Na^+ is considered to be a beneficial element and, to some extent, can replace certain K^+ functions such as an internal osmoticum, in stomatal function, photosynthesis, as a counter-ion in long-distance transport, and in enzyme activation (Marschner 1995; Cramer 1997; Subbarao et al. 2003). However, in glycophytes, elevated Na^+ levels are detrimental, causing specific ion toxicity and negatively affecting root nutrient uptake, especially ions such as K^+ and Ca^{2+} (Verslues et al. 2006).

Sodium influx and toxicity

Land plants do not appear to have specific transport systems for Na^+. Under high external Na^+ concentration, Na^+ enters cells passively via several routes (Cheeseman 1982; Xiong and Zhu 2002). The initial high unidirectional influx of Na^+ into roots is found in species like barley (Kronzucker et al. 2006), wheat (Davenport 1998), maize (Jacoby and Hanson 1985; Zidan et al. 1991) and *Arabidopsis* (Elphick et al. 2001; Essah et al. 2003). This influx is mostly mediated by non-selective cation channels

(NSCCs) (Demidchik et al. 2002b), whose usual function is in the uptake of other cations such as K^+ (Uozumi et al. 2000), and Ca^{2+} (White and Davenport 2002). The unidirectional Na^+ influx rates can exceed net Na^+ uptake rates by an order of magnitude, implying the involvement of high Na^+ efflux (Davenport et al. 1997; Essah et al. 2003).

Na^+ competes with K^+ for binding sites essential for cellular function. With over 50 enzymes activated by K^+, this disrupts numerous enzymatic processes in the cytoplasm (Bhandal and Malik 1988; Marschner 1995). For example, protein synthesis requires high concentrations of K^+ for the binding of *t*RNA to ribosomes, but this is inhibited by high Na^+ *in vitro* (Hall and Flowers 1973; Wyn Jones et al. 1979; Blaha et al. 2000). Enzymes including malate dehydrogenase, aspartate transaminase, glucose 6-P dehydrogenase, and isocitrate dehydrogenase isolated from salt-sensitive broad bean (*Phaseolus vulgaris* L.) and salt-tolerant pop saltbush (*Atriplex spongiosa* F. Muell.) and glasswort (*Salicornia australis* Sol. ex F. Muell.) are equally sensitive to Na^+ up to 500 mM *in vitro* (Greenway and Osmond 1972). Thus, it is essential for plants to employ all sorts of strategies to reduce salt toxicity. Among these, Na^+ exclusion and vacuolar compartmentation are crucial for plant salt adaptation.

Roles of Na^+ exclusion and compartmentation in salt tolerance

Exclusion of Na^+ from the cytosol has been suggested to be a crucial mechanism for salt tolerance in plants (Schubert and Läuchli 1990; Tester and Davenport 2003). Physiological mechanisms of exclusion that operate at the cellular and whole-plant level have been extensively reviewed (Greenway and Munns 1980; Schachtman and Liu 1999; Munns 2002; Véry and Sentenac 2002; Shabala 2003). Ion exclusion mechanisms could provide a degree of tolerance to relatively low concentrations of NaCl but not at high salinity (Yamaguchi and Blumwald 2005). Salt-tolerant wild *Hordeum* species had better Na^+ excluding ability than cultivated barley (Garthwaite et al. 2005). When grown in 50 mM NaCl, bread wheat excluded around 98% Na^+, but barley, durum wheat, and rice excluded about 94% (Munns 2005). There is a strong correlation between salt exclusion and salt tolerance in cereals such as barley, rice and wheat (Flowers and Yeo 1986; Chhipa and Lal 1995; Ashraf and Khanum 1997; Munns and James 2003). In contrast to the Na^+ excluding ability of roots of rice, barley has the ability to prevent root to shoot Na^+ translocation at high external NaCl

(Nakamura et al. 1996). However, a highly salt-tolerant wild relative of tomato (*Lycopersicon esculentum* Mill.) accumulates higher concentrations of Na^+ than the salt-sensitive domesticated tomato (Santa-Cruz et al. 1999). The sensitivity of wild type (WT) *Arabidopsis* and some mutants does not appear to be closely related to shoot levels of Na^+ (Zhu et al. 1998; Nublat et al. 2001; Essah et al. 2003). Therefore, the correlation between plant salt tolerance and its ability to exclude Na^+ from uptake is not straightforward as initially believed.

Compartmentation of Na^+ into the vacuole is also vital for the growth and survival of halophytes and of many non-halophytes, such as barley. Barley is more tolerant to salt than wheat partially due to its greater ability to sequester Na^+ in the vacuole (James et al. 2006). This mechanism would avoid toxic effects of salt on photosynthesis and other key cytosolic metabolic processes (Maathuis et al. 1992; Fricke et al. 1996; Blumwald and Gelli 1997; James et al. 2006). However, such Na^+ compartmentation is not sufficient unless the plant also possesses the ability to efficiently retain K^+ in the cytosol.

2.1.2.2. Potassium

Roles of potassium in higher plants

Potassium is the most abundant cation in higher plants and comprises up to 10% of the total plant dry weight (Marschner 1995). In plants, K^+ plays central roles including osmoregulation, maintenance of turgor pressure, leaf and stomatal movement, enzyme activation, cell elongation, phloem solute transport, cation:anion balancing, control of membrane polarisation, cytoplasmic pH regulation, protein and starch synthesis, and energy conservation across membranes (Wyn Jones et al. 1979; Clarkson and Hanson 1980; Kochian and Lucas 1988; Maathuis et al. 1997; Leigh 2001; Palmgren 2001; Mäser et al. 2002b; Subbarao et al. 2003). The overall contribution of K^+ to the total solute potential, studied in over 200 plant species, varies from 66% to 90% (Wagner 1982; Hsiao and Läuchli 1986). The formation of chloroplast structure and the translocation of assimilates and storage in the sink tissue all critically depend on adequate tissue K^+ concentrations (Flowers and Läuchli 1983). Finally, as discussed above, many metabolic processes and enzymatic reactions in the cytoplasm have a specific requirement for K^+ (Wyn Jones et al. 1979; Marschner 1995).

Effects of salt stress on K^+ homeostasis

K^+ levels in soil solution range from 1 to 10 mM, but intracellular K^+ levels in plants are maintained at 100–200 mM (Kochian and Lucas 1988). Cytoplasmic K^+ levels are well buffered against change by the large vacuolar pool of K^+, but high salt causes a decrease in cellular K^+ concentration and in the K^+/Na^+ ratio (Storey and Wyn Jones 1978). Compared with barley, salt-tolerant wild *Hordeum* species maintain higher leaf K^+ under severe salt treatment (Garthwaite et al. 2005). Salt toxicity on seeds is reflected by decreases in seed K^+ concentration, and leakage of K^+ has been used as a measure of membrane damage from salinity (Nassery 1979; Petruzzelli et al. 1992). Soil salinisation (170 mM NaCl) significantly decreases K^+ concentration in the shoots of salt-susceptible barley cultivars, but the K^+ concentration in the most salt-tolerant one increased (Leonova et al. 2005). Salt treatment also reduces potassium activity (a_K) in the barley epidermal leaf cell vacuoles from 224 to 47 mM and in the cytosol from 68 to 15 mM, while the corresponding changes in the mesophyll were from 235 to 150 mM (vacuole) and 79 to 64 mM (cytosol) (Cuin et al. 2003).

2.1.2.3. Cytosolic K^+/Na^+ ratio is critical for plant salt tolerance

The capacity of a plant to maintain a high cytosolic K^+/Na^+ ratio is one of the key determinants of plant salt tolerance (Serrano et al. 1999b; Amtmann et al. 2004). Under typical physiological conditions, plants contain about 100 mM K^+ and maintain a high K^+/Na^+ ratio in their cytosol (Binzel et al. 1988; Walker et al. 1996), rarely tolerating cytosolic Na^+ levels above 20 mM (Blumwald et al. 2000). *In vitro* activities of enzymes extracted from the halophytes pop saltbush or Seablite (*Suaeda maritima* L.), and even enzymes from the pink salt-lake alga (*Dunaliella parva*) that tolerates 10-fold-seawater salinity, were just as sensitive to NaCl as were those of beans or peas (Greenway and Osmond 1972; Flowers et al. 1977). In contrast to durum wheat, barley is more efficient at Na^+ and K^+ partitioning in cellular and subcellular compartments, leading to the preservation of a higher cytoplasmic K^+/Na^+ ratio at high leaf Na^+ concentrations (Munns et al. 2006). This is supported by electrophysiological analysis of root cation channels: all major K^+ influx channels exhibit higher K^+/Na^+ selectivity in salt cress (*Thellungiella halophila*) than in *Arabidopsis* (Volkov et al. 2004). Rivelli et al. (2002) showed that in low-Na^+ wheat

genotypes, osmotic adjustment was enabled by a higher K^+ concentration, Na^+ exclusion being associated with maintenance of higher K^+ levels. Cytoplasmic Na^+ concentration in salt-sensitive barley was almost 1.4 times greater than that of the salt-resistant one, the latter also having a higher K^+ concentration. It is evident that a salt-sensitive cultivar has a higher Na^+ concentration in its cytoplasm than a salt-resistant variety (Flowers and Hajibagheri 2001).

2.1.3. Interaction between Ca^{2+} and salinity

2.1.3.1. Effect of salinity on Ca^{2+} functions

Calcium is an essential element in all plants. It functions in maintaining the integrity and structure of membranes and cell walls, as a vacuolar counter-cation, and as a second messenger in many signal transduction pathways (Hanson 1984; Bush 1995; Marschner 1995; White and Broadley 2003; Hirschi 2004). Salinity affects Ca^{2+} uptake and transport (Maas and Grieve 1987; Ehret et al. 1990; Munns 2005) at various levels of plant structural organisation. Sodium hampers $^{45}Ca^{2+}$ transport in barley (Lynch and Läuchli 1985), reducing the adsorption of Ca^{2+} on barley root cell walls (Stassart et al. 1981), and disrupting the plasma membrane (PM)-associated and endomembrane-bound Ca^{2+} in protoplasts and vesicles of barley and maize (Lynch et al. 1987; Ehret et al. 1990) with greater effects in salt-sensitive genotypes. The crucial role of Ca^{2+} in plant responses to salinity is further highlighted by discovery of the SOS (Salt Overly Sensitive) pathway. This includes a Ca^{2+}-responsive SOS3-SOS2 protein kinase complex controlling the expression and activity of the SOS1 Na^+/H^+ exchanger (Zhu 2002).

2.1.3.2. High supplemental Ca^{2+} ameliorates Na^+ toxicity

The ameliorative effects of Ca^{2+} on Na^+ toxicity in plants was reported as early as 1902 (LaHaye and Epstein 1969, 1971). It is well-documented that Ca^{2+} reduces Na^+ accumulation in plants (Jacoby and Hanson 1985; Zidan et al. 1991; Fernandez-Ballester et al. 1997; Tester and Davenport 2003). Supplemental Ca^{2+} reduces Na^+ binding to cell walls and the PM (Stassart et al. 1981; Cramer et al. 1985), alleviating membrane leakiness (Cramer et al. 1985), preventing salt-induced decrease

in cell elongation (Kurth et al. 1986), reducing the synthesis of organic metabolites and even restoring the initial growth rate (Fernandez-Ballester et al. 1997). It is reported that external and apoplastic Ca^{2+} directly alleviates symptoms of salt stress through inhibiting NSCC-mediated Na^+ currents (Maathuis and Amtmann 1999; Davenport and Tester 2000; Davenport et al. 2005), thus reducing Na^+ influx into the plant cell.

2.1.3.3. High supplemental Ca^{2+} increases K^+ uptake, helping to maintain high K^+/Na^+ ratio under saline conditions

The K^+ uptake by roots in the absence of Ca^{2+} declines significantly with increasing salinity, but supplemental Ca^{2+} counteracts the unfavourable effect of saline conditions on root K^+ transport, even increasing K^+ uptake (Cramer et al. 1985; Nakamura et al. 1990; Fernandez-Ballester et al. 1997). Ion flux measurements show that the NaCl-induced K^+ loss from the root epidermis can be significantly reduced or even completely prevented by the presence of high external Ca^{2+} concentration, even after several days in saline conditions (Shabala et al. 2003). Calcium ameliorates K^+ loss from the cell by direct and indirect regulation of K^+ efflux channels (Shabala et al. 2006a). The authors found that elevated external Ca^{2+} (up to 10 mM) inhibits Na^+-induced K^+ efflux through two populations of Ca^{2+}-sensitive outwardly-directed K^+-permeable channels in *Arabidopsis* root mature epidermis and leaf mesophyll cells. Thus, supplemental Ca^{2+} helps to establish a favourable intracellular K^+/Na^+ ratio (Fernandez-Ballester et al. 1997; Cramer 2002). Elevated Ca^{2+} mitigates the NaCl-induced inhibition of broomcorn (*Sorghum bicolor* L. Moench) root growth via the maintenance of K^+/Na^+ selectivity (Colmer et al. 1996). In addition to NSCCs, many ion transporters are controlled by Ca^{2+}, either directly or indirectly, via Ca^{2+}-dependent protein kinases, phosphatases or calcineurin-like proteins (Liu and Zhu 1998; Xiong and Zhu 2002).

2.2. ROS stress and synthesis of compatible solutes for plant salt tolerance

2.2.1. Oxidative stress as part of salinity stress

2.2.1.1. ROS and abiotic stress

Major reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot}$), and hydroxyl radicals ($OH^{\cdot}$) (Jakob and Heber 1996; Apel and Hirt 2004). The most common sources of plant ROS are the leakage of electrons to O_2 from the chloroplast and mitochondria, photorespiration in the peroxisomes, and cell wall oxidases and peroxidases (Dat et al. 2000). ROS cause irreversible damage by reacting with various biomolecules and influence the expression of genes and signal pathways. Thus, production of ROS must be strictly controlled (Apel and Hirt 2004). As the equilibrium between production and scavenging of ROS is perturbed by salinity (Tsugane et al. 1999; Hernández et al. 2001), the ability of a plant to cope with increased ROS level under saline condition is important for salt tolerance. In addition to imposing osmotic and ionic stress, high salinity also generates oxidative stress, which is caused by excessive production of ROS (Apel and Hirt 2004; Rodríguez and Redman 2005).

2.2.1.2. Nonenzymatic and enzymatic antioxidants

Nonenzymatic antioxidants include the major cellular redox buffers ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids, and carotenoids (Smirnoff 1993; Apel and Hirt 2004). ROS scavenging enzymes in plants are primarily superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), and glutathione reductase (GR). SOD acts as the first line of defence against ROS, dismutating $O_2^{\cdot}$ to H_2O_2. APX, GPX, and CAT subsequently detoxify H_2O_2 (Apel and Hirt 2004).

Gossett et al. (1994, 1996) found that salinity increased peroxidase (POD) and GR activity in salt-tolerant cotton lines. Under control and saline conditions, a drought-tolerant sorghum variety exhibited efficient H_2O_2 scavenging mechanisms with higher CAT activities than the sensitive one (Jogeswar et al. 2006). Moreover,

the more salt-resistant *Arabidopsis pst1* mutant plants are associated with an increased capacity to scavenge ROS (Tsugane et al. 1999). The activities of SOD, CAT, APX, GPX, and GR were increased significantly in barley roots within 1 d of salt treatment (Sang et al. 2005). Salt-tolerant barley genotypes showed a more rapid increase of POD and SOD activities than the salt-sensitive ones in response to salinity (Huang et al. 2006a). Activities of enzymes involved in ROS scavenging were higher in a salt-tolerant tomato than in its salt-sensitive cultivated relative (Shalata and Tal 1998). It was thus proposed that ROS scavenging activity might be part of the active salt tolerance mechanisms, rather than a secondary response to salinity (Cushman and Bohnert 2000; Tester and Davenport 2003).

2.2.2. Roles of compatible solutes in salinity tolerance

2.2.2.1. Compatible solutes and ROS scavenging

Compatible solutes are non-toxic, highly soluble, neutral or zwitterionic solutes that can be accumulated to high concentrations in the cytosol. They are mainly metabolites such as amino acids (e.g. proline) and sugars (e.g. sucrose, glucose, and fructose), secondary metabolites such as quaternary ammonium compounds (QACs) (e.g. glycine betaine), and polyols (e.g. mannitol, sorbitol, and pinitol) (Yancey et al. 1982; McCue and Hanson 1990; Delauney and Verma 1993; Rhodes and Hanson 1993; Singh et al. 2000). The accumulation of compatible solutes in plants contributes fundamentally to the acclimatisation to salinity (Yancey et al. 1982; Bohnert et al. 1995; Hasegawa et al. 2000; Chinnusamy et al. 2005).

ROS are reported to be scavenged by compatible solutes (Smirnoff and Cumbes 1989; Xiong et al. 2002; Rodríguez and Redman 2005). Sucrose and some polyols (sorbitol, mannitol, and pinitol) are shown to be the most effective ROS scavengers, followed by glutamine and proline. On the other hand, glycines, as well as glycine betaine were shown to be ineffective for hydroxyl radical scavenging *in vitro* (Smirnoff and Cumbes 1989). It should be emphasised that increased salt tolerance of transgenic plants overexpressing variety of compatible solutes (such as mannitol, fructan, trehalose, ononitol, proline, glycine betaine and ectoine) may be due to enhanced oxidative detoxification, as the overall quantities of these substances are

rather low for conventional osmoprotection (Bohnert et al. 1995; Bohnert and Shen 1999).

2.2.2.2. Major compatible solutes and their Reponses to salt stress

Proline

Biosynthesis and function of proline

Proline is primarily synthesised from glutamic acid via Δ-1-pyrroline-5-carboxylate (P5C) by two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR) in plants (Hu et al. 1992; Yoshiba et al. 1997). Proline is known to regulate the accumulation of nitrogen (Stewart and Lee 1974; Ashraf 1994), hydrophobically protect enzymes, membrane stability and cellular structures (Serrano and Gaxiola 1994; Mansour 1998; Gadallah 1999), and limit lipid-peroxidation-linked membrane deterioration by suppressing and detoxifying ROS (McCue and Hanson 1990; Alia et al. 1993; Xiong et al. 2002; Rodríguez and Redman 2005).

Proline in response to salt stress

Proline concentration is usually higher compared to other amino acids accumulating in salt-stressed plants (Ashraf 1994). Its accumulation was found to be negatively related to tissue K^+/Na^+ ratio in salt-treated barley and wheat (Chauhan et al. 1980). In the apices of salt-treated maize seedlings, proline accounted for 50% of the total osmotic adjustment (Voetberg and Sharp 1991). Tobacco cells adapted to NaCl accumulate proline to 80-fold higher than control (Rhodes and Handa 1989). Salinity increased proline and the activity of P5CR in durum wheat, while proline dehydrogenase was inhibited (Mattioni et al. 1997). Exogenous application of proline alleviated the reduction in CAT and POD activities under saline conditions, but did not directly scavenge ROS (Hoque et al. 2007). Accumulation of proline is enhanced in response to salinity in barley, with increased expression of the proline transporter: *Hv*ProT in the root (Ueda et al. 2007). Furthermore, salt treatment induced the specific expression of an *Arabidopsis* proline transporter: ProT2, but the expression of other amino acid transporters decreased, indicating that transport of proline was favoured under stress (Rentsch et al. 1996).

Glycine betaine

Biosynthesis and function of glycine betaine

In plants, glycine betaine (N, N, N-trimethyl glycine) is synthesised from choline involving ferredoxin-dependent choline monooxygenase (CMO) along with Nicotinamide adenine dinucleotide (NAD^+)-dependent betaine aldehyde dehydrogenase (BADH). Salinity induces the activities of both enzymes (McNeil et al. 1999; Nuccio et al. 1999). In salt-stressed plants, glycine betaine (1) stabilises quaternary structures of enzymes, complex proteins and membranes (Gorham 1995; Papageorgiou and Murata 1995; Sakamoto and Murata 2001), (2) protects electron transport via complex II in mitochondria (Hamilton and Heckathorn 2001), (3) destabilises and lowers melting temperature of DNA to promote transcription and replication (Rajendrakumar et al. 1997; Sakamoto and Murata 2001), (4) reduces lipid peroxidation and protects mitochondrial electron transport reactions (Chen and Murata 2002), (5) stabilises the PSII complex (Deshnium et al. 1997; Holmström et al. 2000), and (6) protects Rubisco from inactivation (Nomura et al. 1998).

Glycine betaine in response to salt stress

Many plant species do not accumulate glycine betaine in response to salinity, although barley is one of the natural glycine betaine accumulators (Rhodes and Hanson 1993). The level of accumulated glycine betaine in *Poaceae* species is correlated with the degree of salinity tolerance (Rhodes et al. 1989; Rhodes and Hanson 1993; Saneoka et al. 1995). Genetic evidence that glycine betaine improves salinity tolerance has been obtained for barley and maize (Grumet and Hanson 1986; Rhodes et al. 1989). Exogenous addition of glycine betaine to wheat and maize improved the NaCl-reduced photosynthetic parameters such as P_n, stomatal conductance (g_s), intercellular CO_2 concentration (C_i), quantum yield of photosynthetic electron transport (Φ_{PSII}), and photochemical quenching (qP), but has no effects on maximal quantum efficiency of PSII (F_v/F_m) (Yang and Lu 2005; Raza et al. 2006). In addition, exogenous supply of glycine betaine also increases the salinity tolerance of plants unable to accumulate glycine betaine (Harinasut et al. 1996; Hayashi et al. 1998). For instance, although rice plants do not accumulate glycine betaine, exogenously added glycine betaine improves leaf accumulation of glycine betaine to levels comparable to that in barley and wheat under saline conditions (Harinasut et al. 1996). However,

Jagendorf and Takabe (2001) found stress symptoms, including wilting and loss of chlorophyll, brought on by high NaCl were not necessarily correlated with glycine betaine accumulation. Importantly, transgenic plants engineered to overproduce glycine betaine exhibit moderately or substantially enhanced salinity tolerance even though the levels of glycine betaine are insignificant in the context of osmoregulation (Sakamoto and Murata 2001; Chen and Murata 2002; Wang et al. 2003).

Polyols

Biosynthesis and function of polyols

Polyols are classified as acyclic (e.g. mannitol and sorbitol) and cyclic (e.g. pinitol). Mannitol and sorbitol are direct products of photosynthesis in mature leaves in parallel with sucrose and make up a considerable percentage of all assimilated CO_2 (Noiraud 2001).

Under saline conditions, polyols protect cellular structures by interacting with membranes, protein complexes, or enzymes (Crowe et al. 1992). They associate with protein and membrane components to compensate for water loss (Yancey et al. 1982; Popp and Smirnoff 1995), translocate carbon skeletons and energy between source and sink organs (Noiraud 2001), and function as low-molecular-weight chaperones, and as ROS scavengers (Smirnoff and Cumbes 1989; Bohnert et al. 1995).

Major polyols in response to salt stress

Sorbitol is a direct product of photosynthesis in mature leaves and is found in a variety of plant species, usually as a constituent of seeds (Ahmad et al. 1979). In the halo-tolerant *Plantago maritima*, increasing salinity from 0 to 400 mM resulted in an eight-fold increase of sorbitol concentration in shoots and a 100-fold increase in roots (Ahmad et al. 1979). As the dominant soluble carbohydrate in plantain (*Plantago*) species (halophyte *P. maritima* L. and the nonhalophytes *P. major* L., *P. lanceolata* L., and *P. media* L.), sorbitol increased under saline conditions without significant difference between these species (Königshofer 1983). Moreover, transformants of Japanese persimmon (*Diospyros kaki*) exhibited salinity tolerance by introducing a complementary deoxyribonucleic acid (cDNA) encoding sorbitol-6-phosphate dehydrogenase (S6PDH) (Deguchi et al. 2004).

Mannitol is also a product of photosynthesis in mature leaves (Stoop et al. 1996). Mannitol is synthesised via the action of a mannose-6-phosphate reductase (M6PR) in

celery (*Apium graveolens* L.) shoots (Zhifang and Loescher 2003) and high productivity and high photosynthetic rates in celery are linked to the synthesis of mannitol (Pharr et al. 1995). Binzel et al. (1988) found the protective effect of mannitol as a compatible solute may be sufficient to give a growth advantage in tobacco cells. Mannitol increased significantly in a few *Plantago* species under saline conditions without significant difference between those species (Königshofer 1983). In fully-expanded leaves of olive plants, mannitol increased significantly in saline conditions (Gucci et al. 1998). Recently, Sickler et al. (2007) reported that *Arabidopsis* M2 and M5 transformed with the celery M6PR gene showed significantly higher salinity tolerance than WT. In the presence of 250 mM NaCl, transgenic mannitol-containing tobacco plants have better growth than control plants in terms of better height gain, less fresh weight loss, and more new leaf and root production (Tarczynski et al. 1993).

Halophytes such as mangrove (*Rhizophora mangle* L.) or ice plant (*Mesembryanthemum crystallinum* L.) accumulate pinitol and increase *myo*-inositol methyl transferase (IMT), a key enzyme in pinitol biosynthesis, with the accumulation of Na^+ in ice plant (Dopp et al. 1985; Vera-Estrella et al. 1999). Jojoba (*Simmondsia chinensis*) explants are tolerant to high salinity partly due to increased osmoprotective pinitol concentration (Roussos et al. 2005). In gametophytes of golden leatherfern (*Acrostichum aureum* L.) severe salinity treatment led to a preferential accumulation of pinitol that contributed up to 50% of the soluble carbohydrate pool. The accumulation of pinitol in the gametophytes was significantly correlated with the retention of Φ_{PSII} and its survival in high NaCl (Sun et al. 1999).

Free amino acids

Of the many major solutes contributing to osmotic adjustment in plants subjected to salinity, free amino acids are known to play vital roles (Mansour 2000; El-Tayeb 2005). Salinity increased the size of the free amino acid pool in barley shoots, although it stimulated the reverse trend in the roots (Polonenko et al. 1983). The concentration of free amino acids, including proline increased in barley shoots and roots with increasing level of NaCl (El-Tayeb 2005). Total free amino acids were increased significantly in eight canola (*Brassica napus* L.) lines with increasing external salt concentration and the salt-tolerant line accumulated the highest free amino acids levels at the highest salinity (Qasim 2000). Furthermore, several genes

affecting abiotic stress tolerance are located on chromosome 5A of wheat. Chromosome deletions and salt treatment both affected the amino acid composition (Simon-Sarkadi et al. 2007).

2.3. Ion channels and transporters for plant salt tolerance

2.3.1. Ion transporters for plant salt tolerance

Plant cells employ highly specialised (Doyle et al. 1998; Zhou et al. 2001) primary active and secondary ion transporters to maintain high K^+/Na^+ ratio in the cytosol (Zhu 2003). Precise regulation of ion transport systems is critical for salinity tolerance (Zhu 2003; Chinnusamy et al. 2005). The presence of multiple pathways for uptake of K^+ and Na^+ in plants has long been predicted and K^+ and Na^+ currents of root cells have been intensively studied (Findlay et al. 1994; Gassmann and Schroeder 1994; Maathuis and Sanders 1995; Roberts and Tester 1995, 1997; Tyerman et al. 1997; Amtmann et al. 1997, 1999; Wegner and De Boer 1997, 1999; Demidchik and Tester, 2002; Volkov and Amtmann 2006). Tissue specific transporters for uptake of K^+, export of Na^+, and compartmentation of both ions play important roles in controlling cellular and tissue ion homeostasis (Figure 2.1).

2.3.2. Comparison of ion channels between salt-tolerant and -sensitive genotypes

Differences in ion-selective transport among species and varieties are mostly the result of differences in specific genes including allelic differences and differences in expression, presence or absence of genes (Epstein and Jeffries 1964). Therefore, it is rather tempting to assume that the difference in salt tolerance between species/genotypes may be determined by the difference in major ion transporters at the PM (specifically, those responsible for Na^+ uptake by plant cell). Accordingly, ion transporters of both tonoplast and PM of root cells have been compared between genotypes differing in salinity tolerance (Schachtman et al. 1991b; Findlay et al. 1994

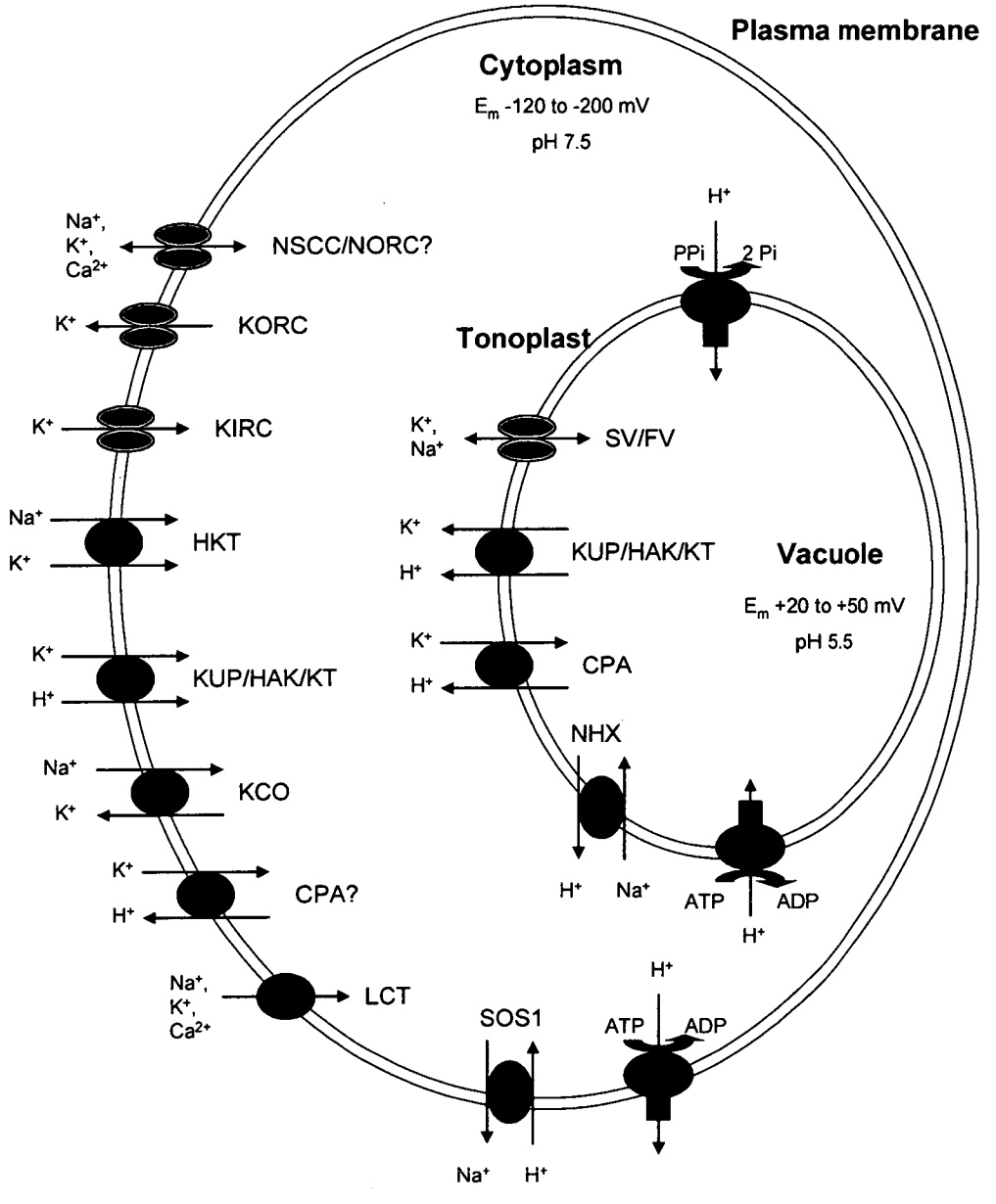

Figure 2.1. Schematic drawing demonstrates a simplified plant cell model representing K^+ and Na^+ transport after salt adaptation. Adapted and modified from Niu et al. (1995), Maathuis and Amtmann (1999), Blumwald (2000), Hasegawa et al. (2000), Mäser et al. (2001), and Shabala (2003).

; Allen et al. 1995), as well as the transport systems of NaCl adapted and non-adapted cells in tissue culture (Niu et al. 1993; Amtmann et al. 1997). However, it was found that the difference in salt tolerance between the two wheat species did not reside in difference in Na^+ transport in PM vesicles (Allen et al. 1995; Davenport et al. 1997). Also, Allen et al. (1995) showed that bread wheat had greater activity of root Na^+/H^+ antiporter than durum wheat when grown in 50 mM NaCl. Schachtman et al. (1991b) examined KORCs and found they had very high selectivity for K^+ over Na^+ in both species of wheat at high and low external Ca^{2+} concentrations. However, Murata et al. (1994) used tobacco suspension cells adapted to 50 mM NaCl to show that the KORCs were considerably reduced in adapted cells. This adaptive change could prevent the K^+ loss under salinity (Cramer et al. 1985). Findlay et al. (1994) found that the properties of potassium inwardly-rectifying channels (KIRCs) and proportions of different types of channels in bread and durum wheat were similar. Furthermore, Amtmann et al. (1997) reported no difference in ion channel properties between salt-adapted and control cells and concluded that adaptation to high salinity did not require the expression of a new type of channel or structural modifications to existing channels. Taken together, these findings emphasise the regulation of channel activity rather than the physical "makeup" of membrane transporters as a key feature of salt tolerance in most species.

2.3.3. Candidate ion channels and transporters

2.3.3.1. Na^+-permeable channels and transporters

One of the important issues to be addressed in research on plant salt tolerance is the identification of channels and transporters responsible for toxic Na^+ influx into the root. Several transporters contribute to Na^+ uptake, but no single gene has been discovered that mediates Na^+ uptake *in planta*. Na^+ may also enter through K^+ transporters that are incompletely selective for K^+ (Amtmann and Sanders 1999; Schachtman and Liu 1999; Mäser et al. 2002b).

NSCCs

It has been proposed that plant Na^+ uptake is primarily mediated by NSCCs (Tyerman and Skerrett 1999; Davenport and Tester 2000; Maathuis and Sanders 2001;

Demidchik et al. 2002b; Essah et al. 2003). These channels are relatively highly selective for Na^+ and are voltage-independent (Maathuis and Amtmann 1999). Radiotracer studies showed multiple kinetic components of Na^+ influx into barley roots (Rains and Epstein 1965, 1967), and Na^+ influx currents have been characterised in electrophysiological studies in root cortex cells of *Arabidopsis* (Demidchik and Tester 2002), wheat (Tyerman et al. 1997; Davenport et al. 2005), maize (Roberts and Tester 1997), and barley suspension cultures and xylem parenchyma cells (Amtmann et al. 1997; De Boer and Wegner 1997). Studies on rye (*Secale cereale* L.), maize, and wheat cortical protoplasts have revealed that external Ca^{2+} blocks Na^+ NSCCs. In maize and wheat protoplasts, Ca^{2+} inhibition of Na^+ influx is 50% of the maximum, indicating that the Na^+-permeable pathway comprises Ca^{2+}-sensitive and -insensitive components (Roberts and Tester 1997; Tyerman et al. 1997; Davenport et al. 2005; Shabala et al. 2006a). On excised patches of maize protoplasts, both current and open probability of NSCCs decrease when the external Ca^{2+} concentration is raised. Furthermore, in both wheat and maize the Ca^{2+}-dependence of the Na^+ currents matches very well the data obtained in $^{22}Na^+$-influx experiments for maize (Zidan et al. 1991) and wheat (Davenport et al. 1997), indicating that Na^+ currents via NSCCs are indeed responsible for the bulk of Na^+ uptake under saline conditions (Tyerman and Skerrett 1999; Davenport and Tester 2000).

NORCs

Non-selective outwardly rectifying cation channels (NORCs) do not discriminate between cations and are activated by increased cytosolic Ca^{2+} concentrations (Wegner and Raschke 1994; Roberts and Tester 1995; Wegner and De Boer 1997). These are another type of K^+ permeable efflux channels present at the PM of barley root epidermal protoplasts. Being non-selective, the NORCs may not only mediate NaCl-induce K^+ efflux, but may also be involved in Na^+ uptake into the root (Wegner and De Boer 1997). All these properties potentially make these channels important to plant salinity tolerance.

Na^+/H^+ antiporters

Plasma membrane Na^+/H^+ antiporter

Plant PM and vacuolar H^+-ATPase constitutes a family of proton pumps driven by the hydrolysis of ATP (Palmgren 2001). Epidermal and endodermal cells of plant roots

have very high amounts of immunodetectable H^+-ATPase (Parets-Soler et al. 1990; Samuels et al. 1992; Jahn et al. 1998). The activity of H^+-ATPases in plants subjected to salinity is still controversial. For example, H^+-ATPase activities in barley roots were higher than those of rice exposed to low (0–25 mM) NaCl concentrations, but in rice activities decreased considerably with an increase of NaCl concentration from 50 to 200 mM, while the activity in those from barley remained high (Nakamura et al. 1996). A decrease in PM H^+-ATPase activity was observed in salt-treated roots of tomato (Gronwald et al. 1990; Sanchez-Aguayo et al. 1991) and buffalograss (Lin and Wu 1996). In contrast, increased H^+-ATPase activity has been reported in culture cells of pea (*Pisum sativum* L.) (Olmos et al. 1993), in a low Na^+-accumulating line of Egyptian clover (*Trifolium alexandrinum* L.) (Parihar et al. 1990), in roots of dwarf saltwort (*Salicornia bigelovii* Torr.) (Ayala et al. 1996), and in cell suspensions of ice plant (Vera-Estrella et al. 1999).

Nonetheless, the activation of proton pumps by salt treatment is positively correlated with salinity tolerance (Golldack and Dietz 2001), and the effect is stronger in salt-tolerant than in salt-sensitive species (Kefu et al. 2003). Furthermore, increase in proton pumping would provide the driving force for a PM Na^+/H^+ exchanger to move Na^+ from the cytoplasm into the apoplast (Ayala et al. 1996).

Na^+ efflux catalysed by the electroneutral plasma-membrane Na^+/H^+ antiporter and encoded by the *SOS1* gene appears to be the main Na^+ efflux system, at least in *Arabidopsis* and rice (Qiu et al. 2002; Shi et al. 2000, 2002; Martinez-Atienza et al. 2007). SOS1 exports Na^+ to the root apoplast and external medium, thus slowing down Na^+ accumulation in the cytoplasm and also influencing long-distance Na^+ transport. SOS1 activity is regulated by a complex composed of the SOS2 kinase and the SOS3 Ca^{2+} binding protein *in vivo* (Zhu et al. 1998; Zhu, 2003). However, this appears to be not the only mechanism controlling the SOS1 antiporter. For example, Shabala et al. (2005b) showed that Na^+ effects on SOS1 may by-pass the SOS2/SOS3 complex in the root apex and that *sos1* mutation affects intracellular K^+ homeostasis by targeting depolarisation activated potassium channels (DAPCs).

Vacuolar Na^+/H^+ antiporter

Vacuolar sequestration of Na^+ is a cost-effective strategy for plant survival in high salinity conditions. As a well-known 'Na^+ includer', barley has high capacity to sequester excess Na^+ in vacuoles. Vacuolar Na^+ sequestration depends on expression

and activity of Na^+/H^+ antiporters as well as the vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase (VPPase), all of which coexist at vacuolar membranes (Apse et al. 1999; Dietz et al. 2001). The gene for the barley vacuolar Na^+/H^+-antiporter (*Hv*NHX2) was identified using the rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) technique. It was shown that the expression of *HVP1* is co-ordinated with that of *HvNHX1* in barley roots in response to ionic and osmotic stresses (Fukuda et al. 2004a). The rate of Na^+/H^+ exchange across tonoplasts was found to increase in response to salinity with possible regulation of *Hv*NHX2 by 14-3-3 proteins (Vasekina et al. 2005). In salt-tolerant barley genotypes, the transport activity of V-ATPase, and protein content of root Na^+/H^+ and K^+/H^+ antiporters: *Hv*NHX2 and *Hv*NHX4, were found to increase after salt treatment. This increase was not measured in salt-susceptible varieties (Ershov et al. 2005, 2007). The organ- and cultivar-specific expression of different isoforms of Na^+/H^+ and K^+/H^+ antiporters indicates that the expression of these isoforms is regulated at the post-transcriptional level during salt treatment (Ershov et al. 2005, 2007).

Transgenic *Arabidopsis* plants overexpressing *AtNHX1* show significantly higher salinity tolerance (200 mM NaCl) than WT plants (Apse et al. 1999). In support of these findings, transferred deoxyribonucleic acid (T-DNA) tagged *atnhx1* plants demonstrated an increase in sensitivity compared to the WT (Apse et al. 2003). Improvement in salinity tolerance evoked by *AtNHX1* overexpression was also observed in canola (Zhang et al. 2001), tomato (Zhang and Blumwald 2001), and rice (Fukuda et al. 2004b). These studies show that overexpression of vacuolar NHX transporters provides an approach that can contribute to molecular breeding of salt-tolerant plants.

2.3.3.2. K^+-permeable channels and transporters

Molecular approaches associated with electrophysiological analysis have greatly contributed to the understanding of K^+ transport in plants. A large number of genes encoding K^+ transport systems have been identified with a high level of complexity (Gierth et al. 1998; Pilot et al. 2003; Véry and Sentenac 2003). Various proteins, such as kinases, phosphatases, 14-3-3 proteins, syntaxins, and farnesyl transferase, are involved in the regulation of K^+ channel activity (Blatt 2000; Assmann 2002; De Boer 2002).

KIRCs and KORCs

Shaker-type KIRCs (Anderson et al. 1992; Sentenac et al. 1992; Maathuis et al. 1997) in the PM of root epidermal and cortical cells are proposed as the main pathway for low-affinity K^+ uptake in plants (Gassmann et al. 1993; Wegner et al. 1994; Maathuis and Sanders 1995). Most KIRCs are highly selective for K^+ over Na^+ and the contribution of KIRCs to low affinity Na^+ uptake is minor (Spalding et al. 1999; Amtmann and Sanders 1999). The *Arabidopsis* K^+ channels AKT1 and KAT1 were the first nutrient ion transport systems identified in plants (Sentenac et al. 1992; Anderson et al. 1992). The K^+ channel AKT2/3 is more relevant to plant salt tolerance because it is largely voltage independent, allows K^+ uptake and efflux, and is up-regulated during salinity (Dreyer et al. 1997; Reintanz et al. 2002; Maathuis 2006). Thus, AKT2/3 has been postulated to function in the recirculation of K^+ through the phloem (Marten et al. 1999).

Shaker-type KORCs have been characterised by patch-clamp experiments in many different plant species (Reviewed by Amtmann and Sanders 1999). The main function of KORCs is assumed to provide a K^+ release pathway, which conducts the majority of NaCl-induced K^+ efflux. KORCs show a high selectivity for K^+ over Na^+ in barley and wheat roots (Schachtman et al. 1991b; Wegner and Raschke 1994; Wegner and De Boer 1997), but a lower K^+/Na^+ selectivity ratio in *Arabidopsis* roots (Maathuis and Sanders 1995). KORCs open during the depolarisation of the PM thus maintaining an outward K^+ current when the less selective cation influx channels allow Na^+ entry (Schachtman et al. 1991b; Maathuis and Sanders 1995). Increasing the external Ca^{2+} from 0.1 to 10 mM leads to a three-fold reduction in the KORC-mediated current in tobacco culture cells (Murata et al. 1998). Based on the typical features of KORC selectivity and gating, KORC do not appear to mediate Na^+-currents (Amtmann and Sanders 1999). No difference was found in KORC properties between salt-sensitive and -tolerant wheat varieties (Schachtman et al. 1991b). Stellar K^+ outward-rectifier (SKOR) and guard cell K^+ outward rectifier (GORK), both of which encode KORCs, are activated upon depolarisation, mediating K^+ efflux (Schachtman 2000). *SKOR* is located at the PM of stellar root tissue where it functions in K^+ release into the xylem and plays an important role in the root-shoot partitioning of K^+ (Gaymard et al. 1998; Pilot et al. 2003) and the SKOR gene is significantly up-regulated during salt stress (Maathuis 2006). GORK has been shown

to be the only outwardly rectifying Shaker channel present in guard cells. *GORK* controls K^+ release from guard cells during stomatal closure (Ache et al. 2000).

HKT

The high-affinity potassium transporters (HKT) are present in most plant species (Véry and Sentenac 2003). Dicots have only one HKT (HKT1;1) and monocots have two HKT sub-families, the first transporting only Na^+ and the second transporting K^+, or Na^+ if the plant is K^+-deficient (Platten et al. 2006; Rodríguez-Navarro and Rubio 2006). High-affinity K^+ uptake is stimulated by micromolar extracellular Na^+, but is blocked at high extracellular Na^+ (Rubio et al. 1995; Gassmann et al. 1996). Decreased expression of HKT under salinity often correlates with plant salinity tolerance (Mäser et al. 2002a; Berthomieu et al. 2003), suggesting that this transporter may be a determinant of salt sensitivity in plants (Rubio et al. 1995). Moreover, *At*HKT1 was identified as a putative regulator of Na^+ influx in plant roots (Rus et al. 2001), and as a mediator of Na^+ loading into the leaf phloem and its unloading from the root phloem sap (Berthomieu et al. 2003; Sunarpi et al. 2005). The rice *Os*HKT1;5 functions as a Na^+-selective transporter in oocytes, and is hypothesised to control shoot Na^+ and influence shoot K^+ by withdrawing Na^+ from the xylem stream into the xylem parenchyma cells (Ren et al. 2005). HKT has been also implicated in the regulation of K^+ transport (Uozumi et al. 2000) and homeostasis (Sunarpi et al. 2005; Ren et al. 2005) under saline conditions in several species, despite HKT transporters only providing a minor component of total K^+ uptake in roots of wheat, barley, and maize (Maathuis et al. 1996; Uozumi et al. 2000; Horie et al. 2001). HKT1 homologs have been isolated from or detected in many species, including *Arabidopsis*, wheat, rice, barley, river redgum (*Eucalyptus camaldulensis* Dehnhardt), and ice plant (Fairbairn et al. 2000; Uozumi et al. 2000; Horie et al. 2001; Garciadeblás et al. 2003; Su et al. 2003).

KUP/HAK/KT

As well as transporting K^+, the KUP/HAK/KT family has also been shown to mediate low-affinity Na^+ influx at high Na^+ concentrations (Santa-María et al. 1997; Vallejo et al. 2005). Genes encoding members of this family were cloned from barley, rice and *Arabidopsis*, and named HAK (Santa-María et al. 1997; Rubio et al. 2000; Bañuelos et al. 2002), KT (Quintero and Blatt 1997), or KUP (Fu and Luan 1998; Kim et al.

1998). Transcript expression of *KUP2* is reported to decrease in shoots of NaCl-treated plants (Maathuis 2006). At the same time, upregulation of ice plant *McHAK1* and *McHAK2* under both K^+ starvation and NaCl stress has been reported (Su et al. 2002). The isoforms are proposed to be involved in maintaining cytoplasmic K^+ levels and in turgor regulation during conditions where external Na^+ inhibits K^+ uptake and replaces K^+. Genes encode KUP/HAK/KT are found in expressed sequence tag (EST) collections from maize, rice, soybean (*Glycine max* L. Merr.), tomato, cotton, onion (*Allium cepa* L.), and ice plant, and they appear to comprise multigene families (Véry and Sentenac 2003; Grabov 2007).

CPAs

A family of cation proton antiporters (CPAs), comprising six putative K^+/H^+ antiporters, has been identified in *Arabidopsis* (Sze et al. 1992; Mäser et al. 2001). Plant K^+/H^+ exchange activity at the tonoplast is expected to be a mechanism for K^+ loading into the vacuole (Apse et al. 1999; Venema et al. 2002). At the PM, the exchange activity contributes to active K^+ secretion into the xylem sap (Kochian and Lucas 1988). Plant K^+/H^+ exchange activity mediated by *At*NHX1, *Le*NHX2, and *Hv*NHX4 was found in addition to Na^+/H^+ exchange in both tonoplast vesicles, (Apse et al. 1999; Zhang and Blumwald 2001; Ershov et al. 2007) and in liposomes reconstituted with purified *At*NHX1 (Venema et al. 2002). The activation of the tonoplast K^+/H^+ exchange may be another factor enhancing plant salinity tolerance because the vacuolar accumulation of K^+ and Na^+ alleviates the osmotic effect of salinity (Ershov et al. 2005), so long as the cytoplasmic K^+ is not depleted. Cellier et al. (2004) suggested that the root and leaf-expressed *Arabidopsis* cation/H^+ exchanger: *At*CHX17 may contribute towards K^+ acquisition and homeostasis under saline conditions, as its transcript level increases under salinity (Maathuis 2006). However, in *chx17* mutants, no difference in Na^+ concentration was observed from wild type plants when grown under saline conditions, while the K^+ levels were decreased in the mutant. This indicates that CHX17 transports K^+ rather than Na^+ during NaCl stress (Cellier et al. 2004).

2.4. Molecular and genetic components of plant salinity tolerance

2.4.1. Genetic diversity of barley salinity tolerance

Breeding for salinity tolerance requires genetic diversity for the character in the gene pool between individual varieties. Understanding the diversity for salinity tolerance in barley will facilitate its use in genetic improvement (Shannon 1997). Many salt-tolerant accessions of barley (Munns et al. 2002) originated from arid, coastal, or saline areas. It is evident that a great magnitude of genetic variation at intraspecific and interspecific levels is present. This can be exploited through selection and breeding for improved salinity tolerance (Al-Khatib et al. 1993; Ashraf and McNeilly 2004). Varietal differences in Na^+ uptake in barley cultivars have been reported (Greenway 1962; Epstein et al. 1980; Richards et al. 1987; Rawson et al. 1988), and there is large variability in salinity tolerance amongst members of the *Hordeum* genus. Halophytic members such as sea barleygrass and foxtail barley (*Hordeum lechleri* Steud. Schenck) are more salt-tolerant than cultivated barley (Garthwaite et al. 2005; Colmer et al. 2006). Large varietal differences for yield in saline conditions have been reported in several studies in glasshouse (Greenway 1962) and field (Richards et al. 1987; Slavich et al. 1990), and also field trials with drip irrigation (Royo et al. 2000). There are also varietal differences in the extent of accumulation of Na^+ in barley leaves (Forster et al. 1994; Colmer et al. 2005).

2.4.2. Genetic approaches to breeding salt-tolerant crops

2.4.2.1. Genetic models for salinity tolerance

Salinity tolerance is a rather complicated trait, both physiologically and genetically. It was reported that barley salinity tolerance at germination is mainly controlled by over-dominant alleles, and non-additive genetic variance is larger than additive genetic variance (Mano and Takeda 1995). Salinity tolerance at the seedling stage (leaf injury index) was reported to be predominantly controlled by additive genes with some effects of dominance, but non-allelic gene interaction was absent (Mano and Takeda 1995, 1997a, b). Similar results in rice based on Na^+, K^+, K^+/Na^+ ratio and sterility (Akbar and Yabuno 1977; Moeljopawiro and Ikehashi 1981; Gregorio and

Senadhira 1993), and in sorghum based on root length (Azhar and McNeilly 1988) were reported with salinity tolerance controlled by both additive and dominant gene effects. In addition, salinity tolerance in sorghum during germination and seedling stages was found to be controlled by complementary gene action, incomplete dominance and dominance or additive effects of several genes (Ratanadilok et al. 1978). Salinity tolerance of maize was under the control of genes with additive and non-additive effects (Rao and McNeilly 1999). The distribution of wheat leaf Na^+ accumulation in individuals in the F_2 population and the $F_{2:3}$ families indicated that three genes of major effect are involved (Munns et al. 2002). Studies on salinity tolerance in different crop species generally show that additive variance is of great value in the improvement of salinity tolerance (Noble et al. 1984; Ashraf and McNeilly 1987).

2.4.2.2. Heritability of salinity tolerance

Breeding procedures depend on the pattern of inheritance, the number of genes with major effects, and the nature of the gene action. Knowledge of the heritability of salinity tolerance is important in determining the selection intensity and number of selection cycles (Downton 1984; Epstein and Rains 1987). In many crop species, high heritability of salinity tolerance is reported. Examples include heritability of seed germination, parent-offspring correlation and selection in barley (Norlyn 1980; Mano and Takeda 1997a,b), Na^+ and K^+ uptake and K^+/Na^+ ratio in rice (Gregorio and Senadhira 1993; García et al. 1997), leaf Na^+ and K^+ accumulation in wheat (García et al. 1997; Munns et al. 2002), shoot growth in lucerne (*Medicago sativa* L.) (Noble et al. 1984; Al-Khatib et al. 1993), root growth in maize (Rao and McNeilly 1999), root length of seedlings in Sorghum (Azhar and McNeilly 1988) and seven grass species (Ashraf et al. 1986), growth in mature plants in intermediate wheatgrass *(Agropyron intermedium* Host P. Beauv.) (Hunt 1965), shoot and root growth in pearl millet (*Pennisetum americanum* L. Leeke) (Ashraf and McNeilly 1987, 1992), and seed germination in alfalfa (Allen et al. 1985).

Norlyn (1980) found that salinity tolerance in barley was heritable, but the genetic control was complex. Highly salt-tolerant varieties were selected from over 6,700 barley accessions. The broad and narrow sense heritabilities of salinity tolerance were as high as 0.99 and 0.75 at germination stage, and 0.85 and 0.75 at

seedling stage, respectively (Mano and Takeda 1997b). Heritability of salinity tolerance in germinating barley seeds was estimated at 0.2 – 0.4 in the F_2-F_3 and 0.8 – 0.9 in the F_3-F_4, indicating higher heritability may be obtained in later generations (Mano and Takeda 1997b). Six basic generations (P_1, P_2, F_1, F_2, BC_1, and BC_2) were employed to investigate genetics of salinity tolerance in wheat using Na^+, K^+ and Cl^- concentration, grain yield and yield components. The high narrow sense heritability (h^2_N) of F_2 and F_1 generations ranged from 0.70 to 0.95 (Ashan et al. 1996), but that of Na^+ and K^+ in salt-stressed rice was only between 0.4 and 0.5 (García et al. 1997).

2.4.3. Molecular markers in breeding for salinity tolerance

Advances in molecular genetic techniques have contributed significantly to better understanding of the genetic, physiological and biochemical mechanisms of plant salinity tolerance. In particular, the development of molecular markers linked with quantitative trait loci (QTL) has, to some extent, improved the efficiency of breeding for salt-tolerant crops (Forster et al. 1997; Flowers et al. 2000). QTLs for salinity tolerance have been described in several cereal species, including barley (Ellis et al. 1997, 2002; Mano and Takeda 1997c), rice (Koyama et al. 2001; Lin et al. 2004) and wheat (Dubcovsky et al. 1996; Lindsay et al. 2004). Barley traits related to salinity tolerance were mapped in a population segregating for a dwarfing gene associated with salinity tolerance (Ellis et al. 1997, 2002), and several QTLs were detected based on the effects of salinity stress on leaf appearance, stem weight prior to elongation, tiller number, grain nitrogen and yield, seedling, growth of leaves and emergence of tillers (Ellis et al. 1997, 2002). Mano and Takeda (1997c) also located barley QTLs for salinity tolerance based on seed viability, germination speed, abscisic acid (ABA) response at germination, shoot length, and leaf injury. In addition, QTLs for Na^+ and K^+/Na^+ discrimination have been shown in rice (Koyoma et al. 2001; Lin et al. 2004). The positions of QTLs controlling salinity tolerance in barley at the seedling stage were different from those at germination (Mano and Takeda 1997c).

2.4.4. Gene expression in response to salinity

A plant's response to salinity is modulated by many physiological characteristics, controlled by the actions of many genes. Substantial progress has been made in the identification of genes with significant effects on plant salinity tolerance (Winicov 1998; Apse et al. 1999; Grover et al. 1999; Hasegawa et al. 2000), and a large number of abiotic stress related genes have also been found to be responsive to salinity in barley (Walia et al. 2006). Transcript regulation in response to high salinity was investigated in rice using microarrays of 1728 cDNAs of rice. Approximately 10% of the transcripts in salt-tolerant Pokkali were significantly upregulated or downregulated within 1 h of salt stress, a response that was delayed in salt-sensitive IR29 (Kawasaki et al. 2001). This complicates the overall search for "salt tolerance genes". Ueda et al. (2002) reported the detection of 133 salt-induced barley cDNA clones that have homology to known proteins. Most genes were expressed strongly in roots. Typical stress tolerance clones encoding glutathione reductase, thioredoxin-like protein, trehalose-6-phosphate synthetase, and heat shock proteins were detected. Genes encoding membrane transporters, and enzymes of sugar or amino acid metabolism were also upregulated. For example, salt treatment of barley increased the root transcript levels of *HVP1*, *HVP10*: two cDNA clones encoding vacuolar H^+-inorganic pyrophosphatase, *HvVHA*: a gene coding for the catalytic subunit of the vacuolar H^+-ATPase, and *HvNHX1*: responsible for vacuolar Na^+/H^+ exchange and leading to vacuolar Na^+ compartmentation (Fukuda et al. 2004a). Furthermore, expression of the genes encoding cell wall proteins (proline rich protein and extensin) and cellulose synthase was induced in barley roots by salt treatment (Ueda et al. 2007). Similar functions or gene names have been detected by comparative analysis of *Arabidopsis* and rice in response to salinity (Rabbani et al. 2003). It will be interesting to compare the salt-induced gene expression in barley with *Arabidopsis* and rice, once the whole barley genome is sequenced.

2.5. Screening methods for salinity tolerance

Over the past few decades, plant breeders have been seeking a reliable and cost-effective screening method for salt tolerance to improve the efficiency in breeding salt-tolerant crops. Screening methods based on physiological traits have

been practically and intensively employed in many plant breeding programmes (Noble and Rogers 1992; Shannon 1997). Despite this, there is still controversy about the use of a simple screening method for salt tolerance and its complex nature. In this section, I list some of those most frequently used screening methods for salt tolerance and discuss their pros and cons.

2.5.1. The complexity of salinity tolerance

Being a polygenic trait, improving plant salt tolerance requires better knowledge of the underlying molecular and physiological mechanisms. Salinity tolerance often shows the characteristics of a multigenic trait (Foolad et al. 2001; Koyama et al. 2001; Flowers 2004; Lin et al. 2004). To add to the complexity, fewer than 25% of the regulated genes are salt stress-specific (Ma et al. 2006), with a multifaceted set of pathways observed in response to salinity. Alterations in the regulation of gene expression and metabolic adjustments in response to salinity share common elements with other abiotic stresses, and it is very difficult to separate these components. It is surprising that, despite the complexity in salinity tolerance, claims are commonly made that transferring or overexpressing a single or a few genes can remarkably improve plant salinity tolerance (Flowers 2004). Indeed, it has been suggested that the overall salt-tolerant trait is determined by several sub-traits, any of which can be determined by many genes whose expression is influenced by numerous environmental factors (Richards 1996; Flowers 2004; Yamaguchi and Blumwald 2005).

2.5.2. Difficulties in screening large numbers of genotypes for salt tolerance

Increasing crop salinity tolerance requires new genetic resources, but breeding efforts are constrained by a shortage of field and laboratory screening tests (Zhu 2000) and unfortunately, few screening procedures have proven successful for identifying salinity tolerance (Shannon 1997) for a number of reasons. Firstly, screening a large number of genotypes for salinity tolerance is difficult due to the complexity and

polygenic nature of salinity tolerance, which involves responses to cellular osmotic, ionic and oxidative stresses (Shannon 1997; Zhu 2000). Secondly, evaluating field performance under saline conditions is difficult because of the variability of salinity within fields (Richards 1983; Daniells et al. 2001) and due to interactions with other environmental factors (Shannon and Noble 1990; Flowers 2004). Thus, physiological traits measured under controlled conditions are employed for rapid and cost-effective selection techniques (Shannon and Noble 1990; Munns et al. 2002). Thirdly, evaluation of the degree of salinity tolerance between and within species is likely to vary according to the criteria used for evaluation (Shannon 1997), so developing an effective screening procedure may in itself be difficult (Flowers and Yeo 1995). Last but not least, evaluating salinity tolerance is made more complicated by the variation in salinity sensitivity at different growth stages: the tolerance of the plants at one growth stage is not always correlated with tolerance at other stages (Greenway and Munns 1980; Flowers 2004; Foolad 2004). Barley has been found to be tolerant to salinity at germination, sensitive at the seedling and early vegetative growth stages, then again tolerant at maturity (Epstein et al. 1980; Munns 2002).

2.5.3. Possible screening methods for salinity tolerance

Physiological traits employed to screen germplasm for plant salinity tolerance have include germination percentage, degree of leaf injury, root length and plant height, shoot and root dry weight, shoot number, maintenance of flowering, seed and fruit set, canopy volume and quality, plant survival under salinity, tissue and specific accumulation of ions in different cell compartments (e.g. Na^+, K^+, Cl^-, Ca^{2+}), or their ratio (e.g. K^+/Na^+, Ca^{2+}/Na^+), and the production of certain metabolites or enzymes (e.g. proline, glycine betaine, sucrose, antioxidative enzymes) (Shannon 1997; Munns et al. 2002; Munns and James 2003; Foolad 2004; Colmer et al. 2005). The use of physiological traits as screening methods has been investigated for rice (Yeo et al. 1990; García et al. 1995) where it has proved successful in generating salt-resistant lines (Gregorio et al. 2002). Thus it may be also applicable to other crops (Cuartero et al. 1992; Ellis et al. 1997; Foolad 1997; Munns et al. 2002). However, Isla et al. (1997; 1998) reported that some physiological traits, including leaf ion concentrations, canopy temperature, stomatal conductance and grain ash content, would not be useful

as screening tools for salinity tolerance in barley, and that grain yield under salinity remains the only reliable measure of identifying salt-tolerant barley.

2.5.3.1. Germination and emergence

Al-Karaki (2001) and Tajbakhsh et al. (2006) showed that salt-tolerant barley maintained a much higher germination rate and shorter germination time than salt-sensitive barley grown under high salinity in Petri dishes. Germination percentage has a strong positive correlation with barley seed and straw yield (Thalji and Shalaldeh 2007). Germination is a convenient test for a large number of genotypes, but little or no correlation has been found between genotypic differences in germination and later growth (e.g. biomass and grain yield) in tolerance to salinity for many species (Kingsbury and Epstein 1984; Ashraf and McNeilly 1988; Mano and Takeda 1997a; Almansouri et al. 2001; Shannon 1997; Munns and James 2003). For example, Donovan and Day (1969) found that a third out of 39 barley cultivars exceeded the germination of the best-known salt-tolerant California Mariout (from which CM72 and Numar originated) under high salinity. Emergence rate has been proposed to be a more practical screening criterion than germination rate (Shannon 1997; Murillo-Amador et al. 2001).

2.5.3.2. Plant growth characteristics in response to salinity

Plant growth components such as plant height, leaf and root elongation rate, and biomass production in saline relative to non-saline conditions are frequently employed as simple and effective screening criteria (Kingsbury and Epstein 1984; Munns and James 2003). Plant height was used as an indicator of salinity tolerance in many species (Joshi and Nimbalkar 1983; Forster et al. 1994; Forster 2001; Houshmand et al. 2005). Seedlings of the salt-tolerant barley cultivar California Mariout showed no growth retardation at 400 mM NaCl, while other genotypes were severely affected at lower salt concentrations (Epstein et al. 1980).

In wheat and maize, it is reported that there are varietal differences in the elongating rate of leaves and roots in early growth responses to salinity (Kingsbury et al. 1984; Mladenova 1990). Aslam et al. (1993) and Moons et al. (1995) showed early varietal difference in rice and subsequent reductions in vegetative and reproductive yields in response to salinity. Moreover, early varietal differences in leaf growth

responses to salinity were detected after only 3 d in two *Brassica* varieties whose leaf Na^+ and Cl^- levels were identical (He and Cramer 1993). These results are only based on observations on plant growth responses to salinity. Will there be any early varietal difference between salt-tolerant and -sensitive barley on the nutrient absorption and salt accumulation immediately after the imposition of salt stress on young seedlings? This question will be addressed in this thesis using a variety of techniques.

Genotypic differences in the effect of salinity on the rate of leaf growth in barley and wheat took a few weeks time to appear (Munns and James 2003). However, within 2 weeks dead leaves became visible on the more salt-sensitive genotype (Munns et al. 1995; Munns et al. 2002). Similarly, two maize cultivars with a two-fold difference in Na^+ accumulation in leaves, showed the same growth reduction. It was not until after 8 weeks that a growth difference was clearly seen (Cramer et al. 1994; Fortmeier and Schubert 1995). Thus, long-term experiments (several weeks to months) are necessary to detect genotypic differences in the effects of salinity on growth in cereal crops such as maize, wheat, barley, and rice (Kingsbury and Epstein 1984; Aslam et al. 1993; Fortmeier and Schubert 1995; Munns et al. 1995; Zhu et al. 2001).

2.5.3.3. Leaf element accumulation

Tissue Na^+ concentration

Leaf Na^+ concentration analysis has the advantage of being directly related to the rate of Na^+ transport to the shoot, thus is specific to Na^+ toxicity in the mesophyll. Na^+ exclusion is frequently used as a screening technique, as genetic differences in exclusion are highly correlated with differences in salinity tolerance between durum and bread wheat (Francois et al. 1986; Gorham et al. 1987). When it comes to barley, Forster et al. (1994) showed that a salt-tolerant mutant was able to limit the amount of Na^+ uptake during salt treatment compared to its isogenic parent. Salt-tolerant barley varieties showed significantly lower Na^+ concentrations than that of salt-susceptible varieties under saline conditions (Tajbakhsh et al. 2006). Extensive screening for salt tolerance in barley, based on Na^+ concentration, has been conducted in thousands of accessions from the world barley collections (Kingsbury and Epstein 1984). Salt tolerance in barley varieties such as CM67 has been correlated with their ability to exclude Na^+ from the shoot (Wyn Jones and Storey 1978; Royo and Aragues 1993, 1999). In addition, leaf Na^+ accumulation has been shown to relate to salt sensitivity

in genotypes of rice, sorghum, and wheat (Yeo and Flowers 1986; Munns 2002; Munns et al. 2006; Krishnamurthy et al. 2007; Thalji and Shalaldeh 2007). It is no exaggeration to say that this trait is the most popular among plant breeders working on improving salt tolerance in plants. There is one hurdle, however, which limits its applicability and reduces the prognostic value of such approach. Tissue Na^+ analysis fails to take into account a plant's ability for Na^+ sequestration, both at the cellular and the tissue level. Therefore, some salt-tolerant genotypes with efficient vacuolar compartmentation (enhanced Na^+/H^+ activity for example) may be simply missed when selection is made based on shoot Na^+ concentration analysis.

Tissue K^+ concentration

Plants that are more tolerant to salt have a greater ability to maintain high levels of K^+. This has been found in crops screened and bred for salinity tolerance, as well as in wild relatives of certain crop species (Colmer et al. 1995; Dubcovsky et al. 1996; Flowers and Hajibagheri 2001; Zhu et al. 2001). For example, Thalji and Shalaldeh (2007) reported that, for barley and wheat, K^+ concentration at the three-leaf stage showed strong positive correlations with seed yield: the ultimate criterion of salinity tolerance.

K^+/Na^+ and Ca^{2+}/Na^+ ratios

The higher Na^+ uptake and lower K^+ concentration exhibited by salt-sensitive barley has been contrasted with lower Na^+ and higher K^+ in salt-tolerant California Mariout and its derivative CM67 (Wyn Jones and Storey 1978; Gorham et al. 1994). Similar results have also been reported by Al-Karaki (2001). Tissue K^+/Na^+ ratio has been used successfully for the selection for salinity tolerance in many crops (Janardhan et al. 1979; Chhipa and Lal 1985; Dvořák et al. 1994; Asch et al. 2000; Tajbakhsh et al. 2006; Thalji and Shalaldeh 2007). Thalji and Shalaldeh (2007) suggested that the barley and wheat K^+/Na^+ ratio can be used as a selection criterion for salt tolerance because it is highly correlated with biomass, seed and straw yields. The Ca^{2+}/Na^+ ratio also appears a more reliable indicator of salt stress than Na^+ concentration alone (Ben-Hayyim et al. 1987; Krishnamurthy et al. 2007). Indeed, the maintenance of higher K^+/Na^+ and Ca^{2+}/Na^+ ratios in young growing tissues does appear to be an important mechanism contributing to improved barley salt tolerance (Wei et al. 2003).

Controversially, bulk leaf ion concentrations of Na^+, K^+, Ca^{2+}, and the K^+/Na^+ and

Ca^{2+}/Na^{+} ratios were not judged to be the cause of the differences in grain yield that were observed in wheat and barley cultivars. Thus it was suggested that these ratios could not be used in screening for salt tolerance (Rawson et al. 1988; Isla et al. 1997). Moreover, Fricke et al. (1996) found that studies using the bulk leaf ion concentrations may be misleading because they do not detect potential ion exclusion mechanisms by the cytoplasm. On the other hand, they showed Na^{+} and Cl^{-} in different leaf compartments could be relevant to barley salt tolerance. However, bulk leaf ion concentrations of Na^{+}, K^{+}, Ca^{2+}, and the ratio of K^{+}/Na^{+} and Ca^{2+}/Na^{+} should be reliable indicators of salt tolerance as shown by their use as some of the most frequent physiological screening methods (Munns and James 2003) and as phenotyping indices in molecular marker studies (Dubcovsky et al. 1996; Koyama et al. 2001; Lin et al. 2004).

2.5.3.4. Photosynthetic parameters

Photosynthesis

Under mild salt treatment (150 mM NaCl), P_n of barley plants was only slightly affected by NaCl treatments (Fricke et al. 1996). However, exposure of a number of barley genotypes to high salinity significantly decreased P_n, g_s, and C_i (Jiang et al. 2006). Furthermore, these responses differed greatly between salt-tolerant and -sensitive genotypes (Huang et al. 2006b). Munns and James (2003) and James et al. (2006) reported that the maintenance of photosynthetic capacity parameters of barley compared to durum wheat at higher leaf Na^{+} levels was associated with the maintenance of higher K^{+}, lower Na^{+} and a resultant higher K^{+}/Na^{+} in the cytoplasm of mesophyll cells in barley. If the major limitation to photosynthesis is stomatal conductance, this parameter may be an effective way of selecting wheat genotypes that will continue to grow in saline soils (Rivelli et al. 2002; Munns and James 2003). However, screening methods based on photosynthesis are not feasible, except for stomatal conductance measured by viscous flow porometery, to handle large numbers (Rebetzke et al. 2000; James et al. 2002).

Chlorophyll fluorescence

Chlorophyll fluorescence was employed for measuring salinity-induced inhibition of PSII (Abadia et al. 1999; Fedina et al. 2002) and thus for laboratory screening of

barley genotypes for salinity tolerance (Belkhodja et al. 1994, 1999). Two-week exposure to 20 dS m^{-1} saline conditions significantly reduced a number of chlorophyll fluorescence parameters such as the Φ_{PSII} and qP in barley (Jiang et al. 2006). In rice (Yamamoto et al. 2004) and wheat (Muranaka et al. 2002) seedlings, Φ_{PSII} markedly decreased for both salt-tolerant and -sensitive genotypes in 100 mM NaCl, but photosynthetic activity was maintained in salt-tolerant lines (Muranaka et al. 2002). Under high salinity, both slowing of electron transport at the PSII in sunflower (Rivelli et al. 2002) and reduced photochemical efficiency of olive (Loreto et al. 2003) have been reported, although neither was associated with photosynthetic reduction. Although F_v/F_m is easy to measure on larger samples, F_v/F_m was not significantly affected in maize by NaCl treatment (Shabala et al. 1998), and F_v/F_m, Φ_{PSII}, qP, and nonphotochemical quenching (NPQ) showed little difference between wheat genotypes in response to 150 mM NaCl, indicating that the efficiency of PSII photochemistry was not affected by salinity (Rivelli et al. 2002). Thus, it is unlikely that chlorophyll fluorescence measurements can be used as a reliable tool in breeding programmes aimed to improve salinity tolerance in plants.

Although measuring gas exchange and chlorophyll fluorescence are not suitable for screening large number of genotypes for salinity tolerance, it is still valuable to investigate effects of salinity on photosynthetic mechanisms in a small number of genotypes.

2.5.3.5. Yield

The grain yield is the ultimate criterion and the main aim of the entire breeding process. It is therefore obvious that grain yield of salt-tolerant barley cultivars is less affected by NaCl than that of the salt-susceptible cultivars (Chauhan et al. 1980; Isla et al. 1998; Flowers 2004; Leonova et al. 2005). Forage yield of Omani Batini barley at tillering stage under 10 and 20 dS m^{-1} salinity can be predicted with high and moderate accuracy by forage yield under 0.85 dS m^{-1} (Jaradat et al. 2004). Importantly, by testing the grain yield of 124 barley genotypes in ten salinity treatments over five consecutive years, Royo and Aragües (1999) found that the most productive genotypes were not necessarily the least salt-tolerant, so it might be useful to select for the most productive barley genotypes under medium and high saline conditions. Also, controlled environment chambers or glasshouses cannot provide adequate space,

light, and pot size required to predict maximum field yield, hence, field evaluation of yield potential under salinity is critical in breeding for salt-tolerant crops. However, field experiments for yield may be more appropriate at the final stages of breeding programmes, rather than at the initial stages when screening for salt-tolerant germplasm is best done under controlled environments (Shannon 1997; Zeng et al. 2002).

2.5.3.6. Survival

Survival under high salinity is also a convenient test of salinity tolerance (Kingsbury and Epstein 1984; Sayed 1985; Tal 1985; García et al. 2002). The rate (percentage) of survival in saline conditions was used to evaluate salt-tolerance in 24 barley genotypes and eight wild *Hordeum* in glasshouse and controlled environment cabinet (Flowers and Hajibagheri 2001; Garthwaite et al. 2005). Furthermore, survival under salinity was employed as a physiological indicator and its QTLs were detected in rice (Lin et al. 2004). The physiological and genetic factors that contribute to growth of crops at very high salt concentrations were found to be proportionally related to survival more than to high yields, despite survival not being of major interest to farmers (Shannon and Noble 1990). Despite showing considerable genetic diversity among 5000 hexaploid and tetraploid wheat lines, only a marginal correlation was found between survival of high salinity and performance in the field (Sayed 1985). Survival at high NaCl as a selection criterion is rapid and simple, but it does not necessarily imply healthy growth and carries the risk of selecting against productivity.

2.5.3.7. Accumulation of compatible solutes

Accumulation of compatible solutes is a typical plant response to salinity exposure (Yancey et al. 1982; Hare et al. 1998). Compatible solute accumulation has long been emphasised as a selection criterion in traditional crop breeding programmes (Morgan 1984; Ludlow and Muchow 1990). The recent progress in molecular biology has made this approach central to molecular breeding programs, largely due to the fact that osmolyte accumulation is often controlled by only a single gene (Serraj and Sinclair 2002). At 300 mM NaCl, glycine betaine and proline together contribute almost 15% to osmotic potential in leaves of halophytic sea barleygrass, compared with only 8% in barley (Garthwaite et al. 2005). Glycine betaine and proline

concentrations in the flag leaf of plants exposed to 200 mM NaCl were much higher in salt-tolerant sea barleygrass than those in salt-sensitive bread wheat, and salinity tolerance was found expressed in their amphiploid (Islam et al. 2007). Due to the controversy over whether high accumulation of compatible solutes is actually beneficial for salinity tolerance in glycophytes, the use of salt-induced compatible solute accumulation is not validated as potential screening tool for salinity tolerance. It also varies among different plant species.

2.5.4. Advances in studying mechanisms of salinity tolerance using the MIFE technique

The proposal to use ion concentrations or electrochemical potentials measured outside plant tissues to calculate tissue flux of the ion came from B. Lucas (Lucas and Kochian 1986). Over the past twenty years, the MIFE technique has been employed in a broad range of research areas in plant sciences (see Newman 2001; Shabala 2006 for reviews), including the response of plants to salinity (Shabala et al. 1998, 2003, 2005a, b), waterlogging (Pang et al. 2006, 2007), Al^{3+} toxicity (Wherrett et al. 2005), Ca^{2+} and Mg^{2+} deficiency (Shabala et al. 2003; Shabala and Hariadi 2005), high and low temperature (Shabala 1996, 1997), rhythmic patterns of nutrient acquisition (Shabala and Knowles 2002; Shabala et al. 2006b), plant response to blue light (Babourina et al. 2002), fluctuations of light intensity (Živanović et al. 2005), and plant ion transporter studies combined with patch-clamp and other techniques for detailed ion transporter studies (Tyerman et al. 2001; Shabala and Lew 2002; Demidchik et al. 2003; Shabala et al. 2006a).

The use of the MIFE techniques has advanced research into plant salinity tolerance (Shabala 2006). For example, bioelectric response measured by MIFE was reported to be a sensitive indicator of NaCl stress in maize leaves (Shabala et al. 1998). Indeed, it has been shown that NaCl stress results in a significant net K^+ efflux (prevented by 10 mM Ca^{2+}) from leaf mesophyll of broad bean (*Vicia faba* L.). In contrast, plants showed a net K^+ uptake in response to isotonic mannitol application. These differences reflect the involvement of both the ionic and osmotic components of salinity stress (Shabala et al. 2000). In suspension cells from wheat, Fusicoccin prevents NaCl-induced K^+ loss from the cell by direct activation of H^+-ATPases and

other metabolic changes crucial for the plant's adaptation to high salinity (Babourina et al. 2000). It was found that there were no effects of NaCl on the net Ca^{2+} flux in protoplasts from broad bean mesophyll, indicating that the large transient NaCl-induced Ca^{2+} efflux from tissue originates from cell wall ion exchange (Shabala and Newman 2000). Shabala et al. (2003) reported that NaCl causes rapid and prolonged efflux of H^+, K^+, and NH_4^+ from the root epidermis with a more positive plasma membrane potential (E_m). The relative efficiency on stimulating barley Na^+ efflux and K^+ uptake was shown to be $Ba^{2+}>Zn^{2+}=Ca^{2+}>Mg^{2+}$ (Shabala et al. 2005b). In addition to their ability to block NSCCs, divalent cations also control the activity of K^+ transporters to maintain the high K^+/Na^+ ratio required for optimal leaf photosynthesis. It has been proposed that compatible solutes prevent NaCl-induced K^+ efflux from barley roots, through enhancing the activity of H^+-ATPase, thereby controlling DAPCs and creating the electrochemical gradient necessary for secondary ion transport processes (Cuin and Shabala 2005). In addition, Cuin and Shabala (2007a) found that a large number of amino acids cause a significant mitigation of the NaCl-induced K^+ efflux from the barley root epidermis, thus suggesting that free amino acids might also contribute to plant adaptation to salinity by regulating K^+ transport across the PM.

The above studies provided insights into the mechanisms possibly responsible for salinity tolerance. However, comparison of salt tolerance between different varieties within the same species is still lacking. Moreover, as a sensitive, non-invasive, and laboratory-based technique, the possibility of using MIFE measurements as a potential screening tool for salinity tolerance has begun to be tested in the studies described in this thesis (Chen et al. 2005, 2007).

2.6. Summary of the literature review

This literature review summarises some aspects of mechanisms for plant salt tolerance, ranging from the whole-plant and cellular to genetic and molecular levels. However, there is still a large gap between theoretical membrane-transport studies at the molecular level and the need for practical screening methods for salt tolerance. Filling this gap is one of the primary aims of this thesis. To achieve this, various physiological, electrophysiological, genetic, and biochemical methods will be

employed to reveal the mechanisms underlying the difference in response to salinity between salt-tolerant and -sensitive barley genotypes. Also, a reliable and efficient screening method based on NaCl-induced ion flux measurement will also be developed, facilitating rapid screening of plant species and cultivars for their salinity tolerance.

Chapter 3. General Materials and Methods

3.1. Plant materials

All the barley genotypes were from the Australian Winter Cereal Collection or from the barley genotype collection of Zhejiang University and Yangzhou University, China. Seeds multiplication of six contrasting genotypes CM72, Numar, ZUG293, Gairdner, Franklin, and ZUG403 were conducted in the field at Mt Pleasant Laboratory, Launceston, Tasmania.

3.2. Glasshouse experiments

Seven glasshouse experiments were conducted in glasshouses at the School of Agricultural Science and the Horticultural Research Centre in the University of Tasmania, Hobart. Two were large-scale screening trials with about 70 barley genotypes tested for their salt tolerance (Figure 3.1; also see 5.2.1), and two were designed to produce F_1, F_2, BC_1 and BC_2 of six barley genotypes (see 6.2.1). Another three experiments were focused on the mechanisms of salt tolerance using a semi-hydroponic system (Figure 3.2; also see 4.2.1 and 8.2.1).

3.2.1. Experiments using the semi-hydroponic system

Eight seeds of each cultivar (7 and 4 genotypes for experiments in chapters 4 and 8, respectively) were sown in a 3-L Polyvinyl chloride (PVC) pot with a mixture containing 70% perlite and 30% sand using half strength Hoagland's solution (HSHS) (Hoagland and Arnon 1938). Plants were watered twice 1 d by an automatic irrigation system through drippers, with about 60 ml solution applied each time per pot (Figure 3.2). A saucer was placed under each pot to retain salt and other nutrients. Plants were thinned to four before the onset of salinity stress.

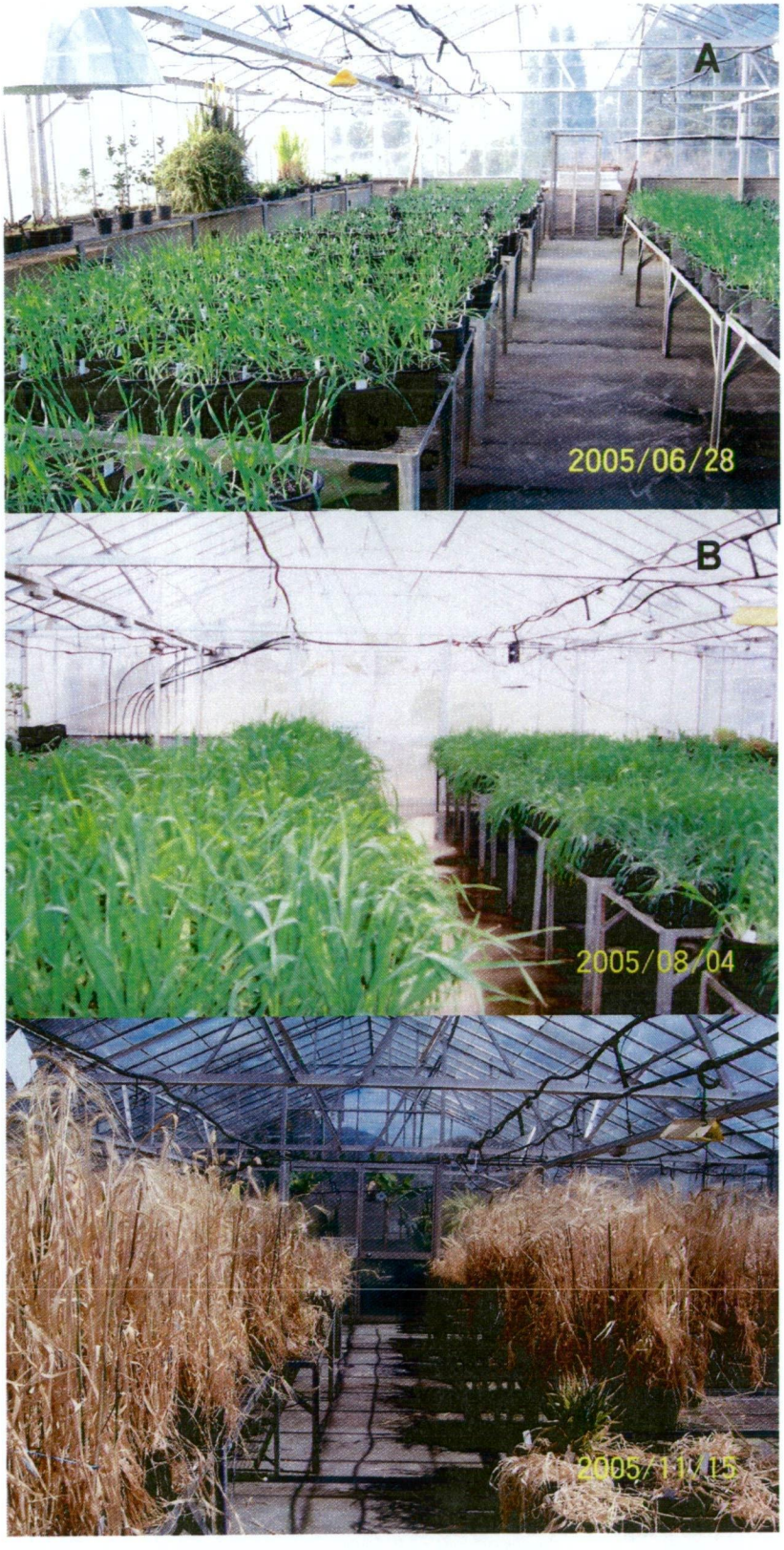

Figure 3.1. Large-scale glasshouse experiments for screening nearly 70 barley cultivars for salinity tolerance. Photos show barley cultivars grown in seedling (A), vegetative (B), and fully mature (C) stages.

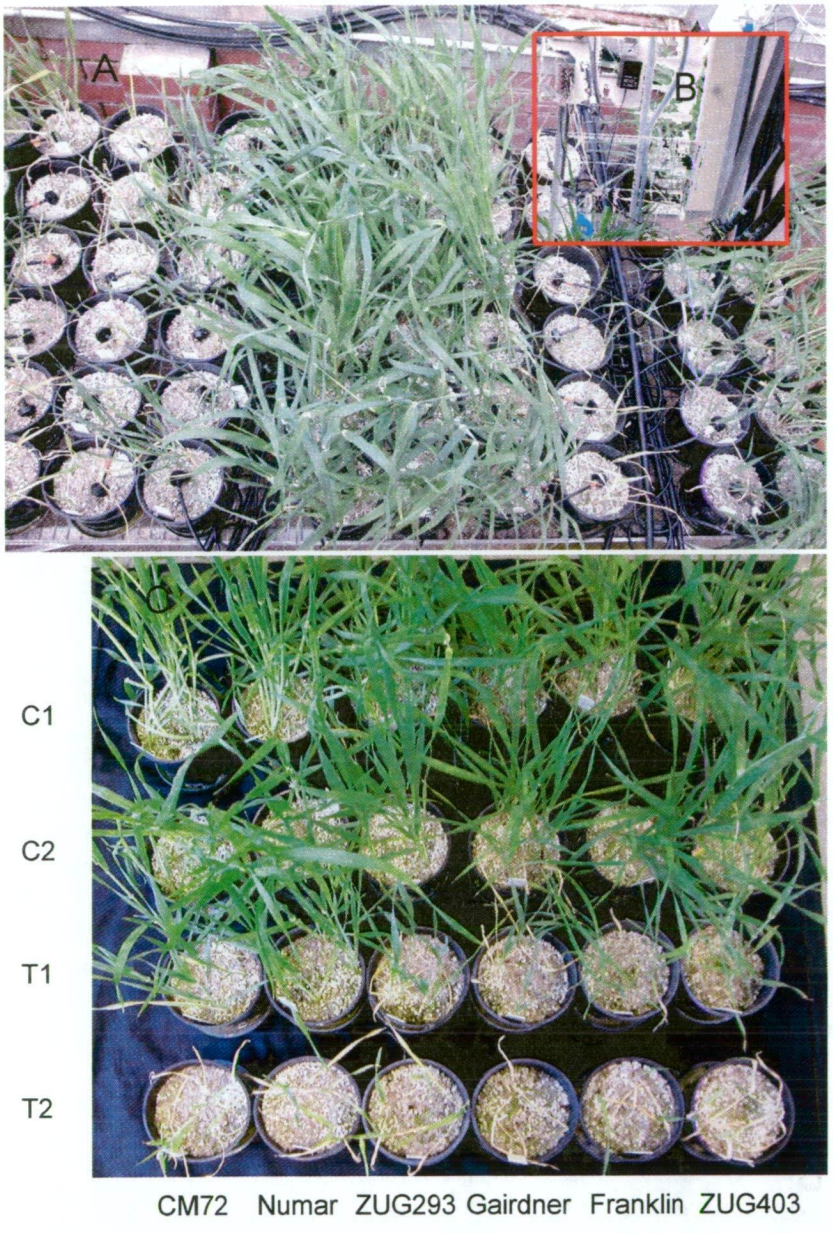

Figure 3.2. Overview of semi-hydroponic glasshouse experiments. (A) – different barley genotypes were grown in a perlite/sand mix and watered with drippers connected to 60-L vials. Irrigation was controlled by a timer (B) connected to all the pipes. For example, six barley genotypes (as marked on panel C) were treated with C1 – half-strength Hoagland's solution (HSHS); C2 – HSHS having only 0.1 mM Ca^{2+}; T1 – HSHS and 320 mM NaCl; T2 – HSHS having only 0.1 mM Ca^{2+} and 320 mM NaCl. Significant differences ($P < 0.05$) were observed between treatments and genotypes (see 7.3).

3.2.2. Large-scale screening

Ten seeds of each cultivar (62 and 69 for Trial 1 and Trial 2 in Chapter 5, respectively) were sown 30 mm deep in a 4.5-L pot and thinned to five healthy seedlings after recording the final germination. The bulk density was about 0.8 kg L^{-1}, and the composition of the potting mix (by volume) was as follows: 80% composted pine bark, 10% sand and 10% coir peat, plus N:P:K (8:4:10), 1 kg m^{-3}; dolomite, 8 kg m^{-3}; wetting agent, 0.75 kg m^{-3}; sulphate of iron, 1 kg m^{-3}; gypsum, 1 kg m^{-3}; Isobutylenediurea (IBDU), 1 kg m^{-3}; trace element mix, 0.75 kg m^{-3}; zeolite, 0.75 kg m^{-3}; pH 6.0.

3.2.3 Producing barley hybrids

To produce barley hybrids for genetic studies on salt tolerance, parental lines (CM72, Numar, Yu6472, Yan90260, Gairdner, and Franklin) were grown in 8-L pots with standard potting mix for crossing to produce F_1 hybrid seeds. Seeds of the six cultivars were sown on different dates to give sufficient overlap in flowering for artificial emasculation and cross pollination. Each cultivar had 4 replicates with 10 seeds sown in each pot. General irrigation and pest controls were applied. After harvesting, K^+ flux was measured from roots of half of the F_1 seedlings and they then grown for F_2 in a glasshouse at the School of Agricultural Science in Hobart. The rest were sown in a glasshouse at Mt Pleasant Laboratory in Launceston to produce F_2, BC_1, and BC_2 of CM72/Gairdner and CM72/Franklin.

3.3. MIFE

3.3.1. MIFE theory and system

The theory of the MIFE technique was reviewed in detail by Newman (2001). Briefly, if an ion is taken up by plant cells, its concentration in the proximity of the cell or tissue surface will be lower than that in solution further away (Figure 3.3). The

principle of the MIFE technique is to measure this electrochemical potential gradient by slow square-wave movement of ion-selective electrodes between two positions, close to (position 1), and distant from (position 2) the sample surface (Figure 3.3). At each position, electrode voltage is recorded and then converted into concentration using the calibrated Nernst slope of the electrode. It is assumed that convection and water uptake are negligibly small and unstirred layer conditions are met (see Newman 2001; Shabala 2006 for reviews). The MIFE system has been developing for the last two decades with the advances in microscope, micromanipulator, and computer technology (Figure 3.4 shows the MIFE system with some of the latest components).

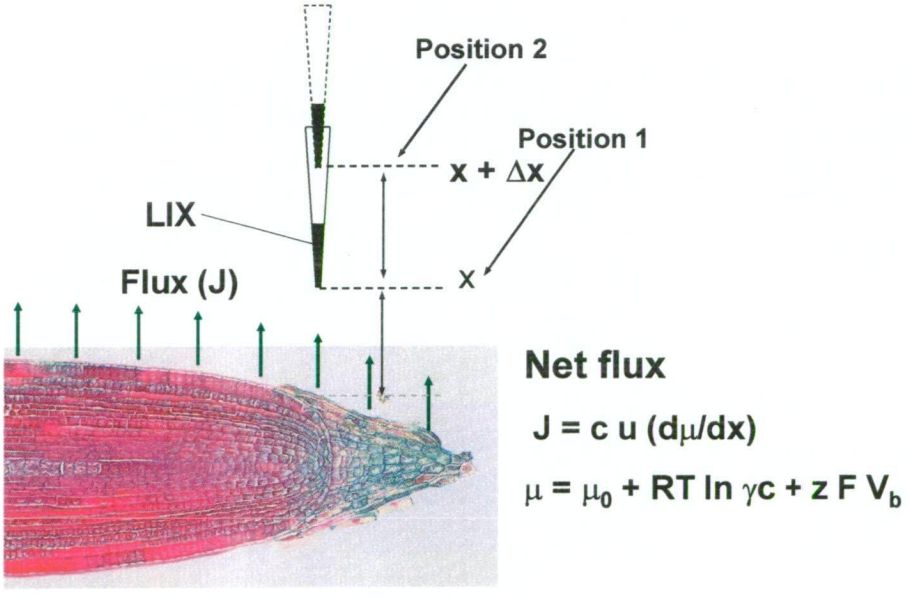

Figure 3.3. Basic principle of the MIFE measurement. The ion-selective microelectrode is moved in a square-wave manner between two positions near the root surface. Adapted from Wherrett (2006).

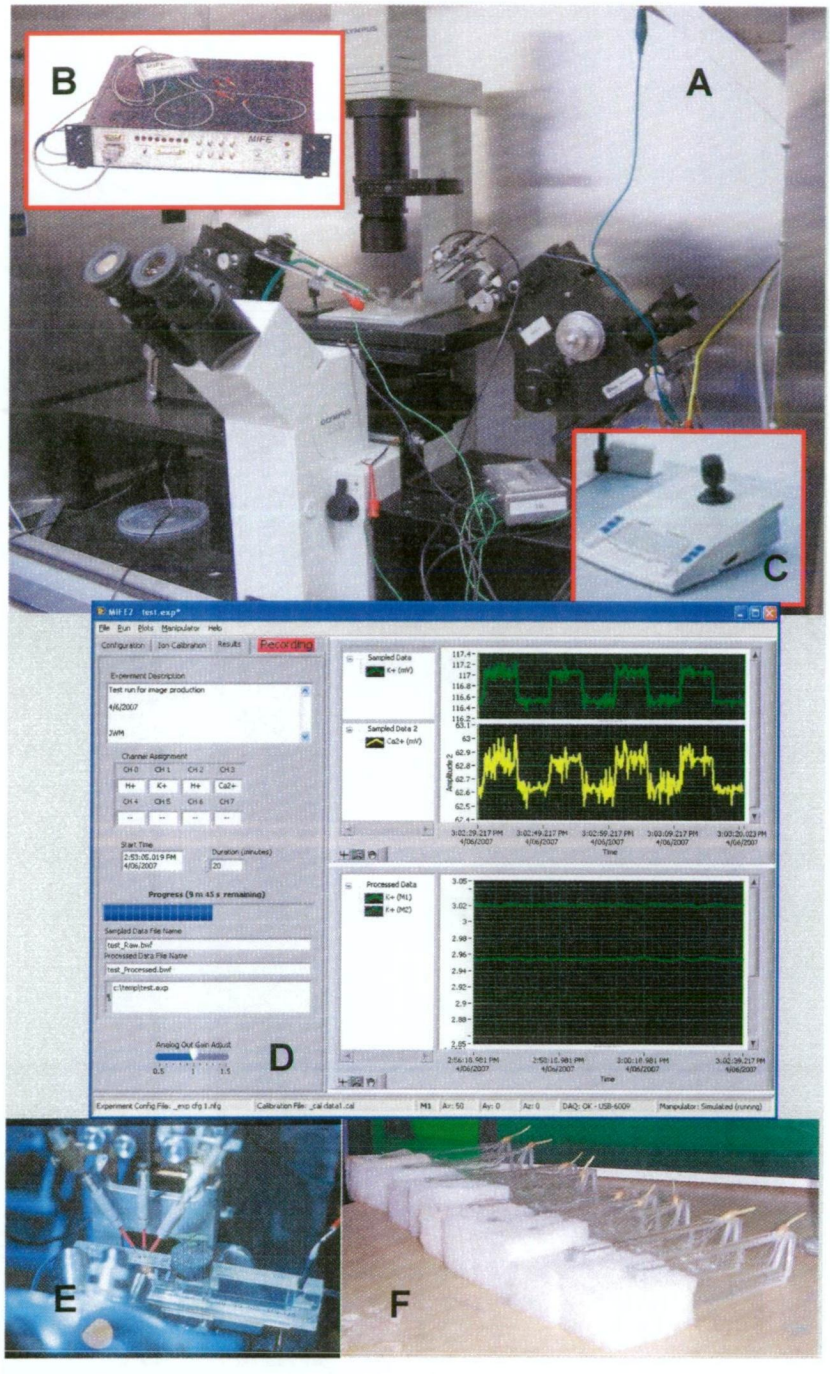

Figure 3.4. Overview of a MIFE system. It consist an inverted microscope (A), MIFE amplifier and preamplifier (B), micromanipulator Patchman NP 2 (C), and a standard computer with MIFE software (D). In panel A, MIFE flux measurements (right) and patch-clamping (left) are undertaken simultaneously on a protoplast. For seedling roots, it is more convenient to use a standard compound microscope lying on its back, with the root in a vertical chamber (E). In the current screening experiments, barley seedlings were pretreated with 80 mM NaCl in 10 ml measuring chambers for 1 h before 5 min of K^+ flux measurement (F).

3.3.2. Ion flux measurements

For ion flux experiments and for measurements of K^+ leakage from the root, seedlings were grown for 3 d in an aerated hydroponic solution (0.5 mM KCl and 0.1 mM $CaCl_2$) in a dark growth cabinet at 24 ± 1°C. Seedlings with root length of 70 ± 10 mm of all the cultivars and crossing lines were used for K^+ flux measurements. Most ion flux measurements were undertaken non-invasively on the root mature zone ~10 mm from the root tip using the MIFE technique (University of Tasmania, Hobart, Australia) as described in previous publications (Shabala et al. 1997, 2003). One hour prior to measurement, a seedling was taken from the growth cabinet and placed in a 10-ml Perspex measuring chamber with 10 ml 80 mM NaCl, which contained 0.5 mM KCl and 0.1 mM $CaCl_2$. The use of low Ca^{2+} concentrations was required to increase the sensitivity of the method (to maximise the difference in ion flux responses between salt-sensitive and -tolerant genotypes). This issue is addressed in detail in Chapter 8. The root was centred within the chamber and fixed horizontally by immobilising the root using moveable plastic cross-bars within the chamber (Shabala et al. 1997; Chen et al. 2005). Electrodes were filled with ion-selective cocktails (ionophore Na^+ 71178; K^+ 60031; H^+ 95297; Ca^{2+} 21048; Fluka, Buchs, Switzerland), and their tips aligned and positioned 40 μm above the root surface. During measurements, electrodes were moved in a slow (10-s cycle, 40-μm amplitude) square-wave by a computer-driven micromanipulator (Patchman NP2, Eppendorf, Hamburg, Germany) between the two positions, close to (30–40 μm) and away from (70-80 μm) the root surface. Net ion fluxes were calculated from the measured difference in electrochemical potential for these ions between two positions using cylindrical diffusion geometry (Shabala et al. 1997; Newman 2001).

3.4. Measurements of physiological parameters

3.4.1. Growth components

Plant height from all the large-scale glasshouse experiments and semi-hydroponic trials were measured before harvesting. Shoot and root fresh weight was measured

immediately by a Mettler BB2440 Delta Range balance (Mettler-Toledo, Greifensee, Switzerland). Shoots and roots were then dried at 65°C in a Unitherm Dryer (Birmingham, UK) to constant weight and weighed again.

3.4.2. Na^+ and K^+ concentrations

Leaves and roots, harvested and dried at 65°C to constant weight, were then ground and passed through a 2-mm mesh sieve. A sample of ~ 0.3 g was collected and digested in 10 ml 98% H_2SO_4 and 3 ml 30% H_2O_2 for 5 h essentially as described by Skoog et al. (2000). The Na^+ and K^+ concentrations were determined by a flame photometer (Corning 410C, Essex, UK). K^+/Na^+ ratio was then calculated.

3.4.3. Leaf sap osmolality

One day prior to harvest for biomass, four segments from flag leaf blades for each treatment were sampled and immediately preserved at -20°C. Flag leaf blade sap was extracted using the freeze-thaw method (Tomos et al. 1984) and its osmolality was determined using a vapor pressure osmometer (Vapro, Wescor Inc. Logan, Utah, USA).

3.4.4. CO_2 assimilation

Measurement of P_n was undertaken on barley flag leaves using an LCi portable infrared gas analyser (ADC BioScientific, Hoddesdon, UK) on clear days. The background illumination of 800 ± 20 μmol photons m^{-2} s^{-1} was provided by a metal halide lamp (OSRAM 400W, Eichstätt, Germany). A water-cooling and circulation system was placed between the plants and light source to prevent heat damage to the plants. The sample size was four replicates for each cultivar and treatment.

3.5. Statistical and genetic analysis

Data were analysed using SPSS 14.0 for Windows (2005). All the results are given as means ± SE. Significance of differences was determined by Student's *t*-test. Different *lowercase* letters indicate significance at either $P < 0.05$ or 0.01 levels. In Chapter 6, parental lines and F_1s were subjected to an analysis of variance, and the validity of the additive-dominance model was assessed using joint regression Wr/Vr analysis, the *A*, *B*, and *C* scaling test and the joint scaling test according to Mather and Jinks (1977).

Chapter 4. Screening Plants for Salt Tolerance by Measuring K^+ Flux: a Case Study for Barley

4.1. Introduction

The bottleneck of any plant breeding programme is the lack of reliable, convenient, inexpensive and quick screening techniques (Zhu 2000; Munns and James 2003). In most cases, field screening for salinity tolerance remains the main tool, despite its limitation of time requirement and environment dependence. Many potential criteria or traits have been proposed for screening. Examples include ranking of plants according to growth rate or yield (Greenway 1962), plant survival at high salinity (Sayed 1985), germination rate (von Well and Fossey 1998), leaf or root elongation rate (Cramer and Quarrie 2002), leaf injury and reduction of P_n (James et al. 2002), loss of chlorophyll and damage to the photosynthetic apparatus (Krishnaraj et al. 1993), Na^+ exclusion (García et al. 1995), K^+/Na^+ discrimination (Asch et al. 2000) and Cl^- exclusion (Rogers and Noble 1992). Many of these criteria are often unrelated to each other, resulting in different estimates of salt tolerance. As a multigenic trait, salt tolerance involves responses to cellular, osmotic and ionic stresses and their consequent secondary stresses and whole-plant coordination. Hundreds of different genes may be involved, either directly or indirectly. Some of these genes are expressed at very early stages, while others become crucial only at later stages of plant ontogeny. All this complicates plant screening for salt tolerance, and crop ranking made at one stage may be rather different from similar assessment made at another stage of plant ontogeny. Obviously, knowledge of underlying physiological mechanisms is of paramount importance for efficient screening methods (Zhu 2000). Some researchers (Shannon and Noble 1990; Flowers and Yeo 1995) have suggested that screening for salt tolerance be carried out using physiological markers, or that physiological traits should be used as selection criteria, either singly or in combination, rather than selection being simply upon yield or yield components.

One of the key features of plant salt tolerance is the ability of plant cells to maintain optimal K^+/Na^+ ratio in the cytosol (Maathuis and Amtmann 1999; Tester and Davenport 2003). In normal conditions, with cytosolic K^+ being around 150 mM (Wyn Jones et al. 1979; Leigh and Wyn Jones 1984) and cytosolic Na^+ in a much

lower range (Carden et al. 2003), this ratio is rather high (~ 100), enabling normal cell metabolism. The latter includes cell osmoregulation, turgor maintenance, stomatal function, activation of enzymes, protein synthesis, oxidative metabolism, and, in particular, photosynthesis (Marschner 1995; Shabala 2003). Under salinity, however, the K^+/Na^+ ratio falls dramatically (Maathuis and Amtnann 1999). This occurs as a result of both excessive Na^+ accumulation in the cytosol (Leigh 2001; Zhu 2000) and enhanced K^+ leakage from the cell (Shabala 2000; Shabala et al. 2003); the latter resulting from NaCl-induced membrane depolarisation under saline conditions (Cakirlar and Bowling 1981; Shabala et al. 2003). It is not surprising therefore that the K^+/Na^+ ratio in plant tissues has often been suggested as a potential screening tool for plant breeders (Shannon 1997; Poustini and Siosemardeh 2004).

However, there appears to be some confusion between cytosolic K^+/Na^+ ratios and K^+/Na^+ ratios in salinised plant tissues (e.g. roots or shoots), which is what most breeders refer to. The latter ratio fails to take into account the fact that a significant part of accumulated Na^+ may be compartmentalised in the vacuole. Vacuolar compartmentation is another key feature of plant salt tolerance (Blumwald 2000). Unfortunately, traditional tissue analysis for Na^+ concentration, based on acid tissue digestion followed by AA (atomic absorption) -spectroscopic analysis, which is used as a basis to determine K^+/Na^+ ratio in plant tissues, cannot account for such compartmentation. This diminishes the predictive value of the K^+/Na^+ ratio in plant tissues to screen plants for salt tolerance.

Being both technically challenging and expensive, X-ray analysis, usually used to determine cytosolic K^+/Na^+ ratios (Flowers and Hajibagheri 2001) is not applicable as a screening tool. How then can the problem be tackled? As a viable alternative, non-invasive methods to quantify net fluxes of ions into and out of plant tissues may be employed. There is evidence that a cell's ability to retain K^+ is at least as important for plant salt tolerance as its ability to exclude or compartmentalise toxic Na^+ (Shabala 2000; Shabala et al. 2003). On this basis K^+ uptake measurement may provide a quick and reliable screening test on seedlings that will save field space and time. The above hypothesis was comprehensively tested in this study, by applying the MIFE technique (Shabala et al. 1997; Newman 2001) to measure non-invasively specific ion fluxes in solution near roots of a range of barley cultivars differing in their salt tolerance. Based on data presented here, a relatively quick and reliable

method is proposed, to screen plants for salt tolerance using non-invasive K^+ flux measurements.

4.2. Materials and methods

4.2.1. Plant materials and growth conditions

Seven barley cultivars (CM72, Numar, Franklin, Gairdner, ZUG293, ZUG95, and ZUG403) were used in this study. Barley seeds were surface sterilised with 3% H_2O_2 for 10 min and thoroughly rinsed with distilled water. Whole-plant responses to salinity were studied in glasshouse experiments, using a semi-hydroponic culture technique. After 3 weeks, seedlings were thinned to leave four uniform and healthy seedlings in each pot. Salinity treatment, in HSHS, was applied to 3-week old plants and lasted for five weeks. During the first 2 weeks of the salt stress, NaCl treatments were 0 (control), 80 and 160 mM; during the last three weeks, NaCl concentrations were doubled to 0, 160 and 320 mM, respectively. These treatments are referred to as low (0 mM), moderate (160 mM) and severe (320 mM) salt stress throughout the text. A randomised complete block design was used, with four replications for each treatment.

4.2.2. Biomass

Plants were harvested at the age of 8 weeks, after 5 weeks of NaCl treatment. Pots were soaked in barrels with tap water for five min. Perlite and sand particles sticking to the roots were gently removed, and then roots were thoroughly rinsed with tape water. Each plant was separated into shoots and roots, excess water removed by blotting roots with paper towels. Measurements of plant height and fresh and dry weight were described at 3.4.1. Shoot or root water content (%) was calculated as the difference between fresh and dry weight of the roots or shoots.

4.2.3. Net CO_2 assimilation and chlorophyll fluorescence

P_n was measured on flag leaves at weeks 2, 3, 4, and 5 after NaCl treatment

commenced (see 3.4.4 for details). After measuring P_n, the chlorophyll fluorescence was recorded from intact flag leaves on the same dates using a portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) essentially as described by Smethurst and Shabala (2003). Two measurements were undertaken in each pot for both photosynthetic and chlorophyll fluorescence parameters.

4.2.4. K^+ leakage from the roots

Seedlings of four barley cultivars (CM72, Numar, Franklin, and Gairdner) were grown essentially as described for ion flux experiments (see 3.3.2). Thirty uniform 3-d old seedlings for each cultivar were chosen and divided into 10 batches. Roots were immersed in a 25-ml beaker (3 seedlings per beaker) with 10 ml 80 mM NaCl and kept there for 2 h. After that, seedlings were removed, their roots blotted dry by paper towel, and root fresh weight measured. The amount of K^+ released into solution was determined using a flame photometer.

4.2.5. Ion flux measurements

Details of flux measurements of Na^+, K^+, H^+, and Ca^{2+} are in 3.3.2.

4.2.6. Experimental protocols for MIFE measurements

Transient ion flux kinetics. Net ion fluxes were measured for 10 min in control (bath solution) to ensure steady initial values. Then salinity treatment (20, 40, 80 or 160 mM; applied as the double stock made up in 5 ml of the bath solution) was given, and transient ion flux responses were measured for another 50 min. When mannitol was used instead of NaCl to mimic the hyperosmotic stress imposed by salinity, 35 or 140 mM mannitol concentrations (isotonic to 20 and 80 mM NaCl, respectively) were used.

Steady-state measurements. Seedlings were pre-treated with NaCl for 80, 150, and 300 min, and K^+ flux was then measured for 30 min. For the 5-h treatment, the solution was replaced by fresh solution at 120 and 240 min.

Measure K^+ flux along the root. K^+ flux profiles along the root axis were measured in control and after 1 h of incubation in 80 mM NaCl-containing solution. Root scanning commenced from the tip and was carried out with 0.6 mm increments, with net ion fluxes measured for 60 s at each position.

Measure K^+ flux from roots of different age. Seedlings of two cultivars, CM72 and Gairdner, were grown for 2, 3, or 4 d. K^+ fluxes were measured at about 10 mm from the root tip for 10-15 min after 1 h pre-treatment in 80 mM NaCl.

4.3. Results

4.3.1. Whole-plant responses

4.3.1.1. Biomass

Five weeks of salinity treatment had a strong impact on root and shoot growth, with both fresh and dry weight significantly ($P < 0.05$) reduced (Table 4.1). The impact of salinity, however, differed substantially between barley cultivars. Based on this data, the most salt-tolerant are CM72, Numar and ZUG293 (on average, 83 and 56% of control root dry mass at intermediate and severe salt stress, respectively). Franklin, Gairdner and ZUG403 cultivars, on the contrary, showed much greater sensitivity to NaCl treatment (48 and 27% of control at intermediate and severe salt stress, respectively). In general, shoot biomass was less affected, with salt-tolerant cultivars showing no statistically significant reduction in shoot dry weight for intermediate (160 mM NaCl) treatment (Table 4.1). Shoot fresh weight for this treatment, however, was significantly ($P < 0.05$) reduced (67% of control for the three salt-tolerant cultivars; Table 4.1).

4.3.1.2. Photosynthetic characteristics

Salinity caused a significant ($P < 0.05$) reduction in P_n (Figure 4.1A), with the effect being proportional to both severity of the salt stress (Figure 4.1A) and duration of the treatment (data not shown). After 5 weeks of treatment, salt-tolerant varieties maintained P_n 2 to 3-fold higher than the salt-sensitive ones for severe (320 mM NaCl) treatment (Figure 4.1A). The difference is significant at $P < 0.05$. At the same time, no

apparent impact of salinity on chlorophyll fluorescence characteristics was found (Figure 4.1B). Regardless of the severity of the salt stress and genotypic difference between cultivars, maximum photochemical efficiency of PSII (F_v/F_m value) remained above 0.8, indicating the absence of detrimental salinity effects on leaf photochemistry (Figure 4.1B).

Table 4.1. Root and shoot biomass of seven barley cultivars after 5 weeks exposure to various NaCl levels. Means ± SE (n = 16).

	Treatment/	*Fresh weight, g plant^{-1}*			*Dry weight, g plant^{-1}*		
	Cultivar	0 mM	160 mM	320 mM	0 mM	160 mM	320 mM
Root	CM72	4.1±0.5	2.7±0.3	1.8±0.2	0.27±0.03	0.21±0.02	0.15±0.01
	Numar	3.1±0.2	2.5±0.1	1.7±0.1	0.19±0.01	0.17±0.01	0.10±0.01
	ZUG293	3.5±0.2	2.8±0.1	1.6±0.1	0.24±0.01	0.19±0.02	0.13±0.01
	ZUG95	4.6±0.4	2.2±0.1	1.1±0.1	0.31±0.02	0.18±0.01	0.09±0.01
	Franklin	3.2±0.1	1.5±0.1	1.1±0.1	0.20±0.01	0.09±0.01	0.07±0.01
	Gairdner	3.6±0.2	1.8±0.2	1.0±0.1	0.26±0.02	0.13±0.01	0.06±0.01
	ZUG403	4.2±0.3	1.9±0.1	1.0±0.1	0.31±0.02	0.15±0.01	0.08±0.01
Shoot	CM72	9.1±1.1	6.7±0.9	3.5±0.3	0.83±0.08	0.87±0.10	0.43±0.03
	Numar	11.2±0.6	6.7±0.3	3.3±0.2	0.86±0.04	0.85±0.04	0.44±0.03
	ZUG293	9.4±0.6	6.4±0.4	3.4±0.2	0.83±0.05	0.89±0.08	0.44±0.03
	ZUG95	12.0±0.8	5.4±0.3	2.2±0.1	0.97±0.05	0.68±0.04	0.35±0.02
	Franklin	9.4±0.4	4.1±0.3	1.8±0.2	0.76±0.03	0.53±0.02	0.27±0.03
	Gairdner	8.5±0.5	4.6±0.5	0.8±0.1	0.80±0.06	0.60±0.07	0.14±0.02
	ZUG403	11.3±0.8	5.1±0.4	1.0±0.1	0.97±0.07	0.49±0.04	0.17±0.02

4.3.1.3. Water content and leaf sap osmolality

No clear trend was observed in effects of elevated NaCl levels on root water content (Figure 4.2A). Shoot water content (SWC), however, was significantly ($P < 0.05$) reduced by salinity, with the effect increasing with the severity of salt stress (Figure 4.2B). A significant ($P < 0.05$) difference in SWC between salt-tolerant (CM72,

Numar and ZUG293) and salt-sensitive (Gairdner and ZUG403) cultivars was found for the 320 mM NaCl treatment (Figure 4.2B). Changes in SWC were mirrored in changes in the leaf sap osmolality (Figure 4.2C), with plants doubling (compared with control) sap osmolality of the flag leaf at 160 mM treatment. No significant genotypic difference was observed, however, for this treatment (Figure 4.2C). More severe treatment (320 mM NaCl) not only caused a further increase in leaf sap osmolality, but also allowed a clear differentiation between genotypes according to their salt tolerance, with about a 2-fold difference between salt-sensitive and -tolerant genotypes (Figure 4.2C).

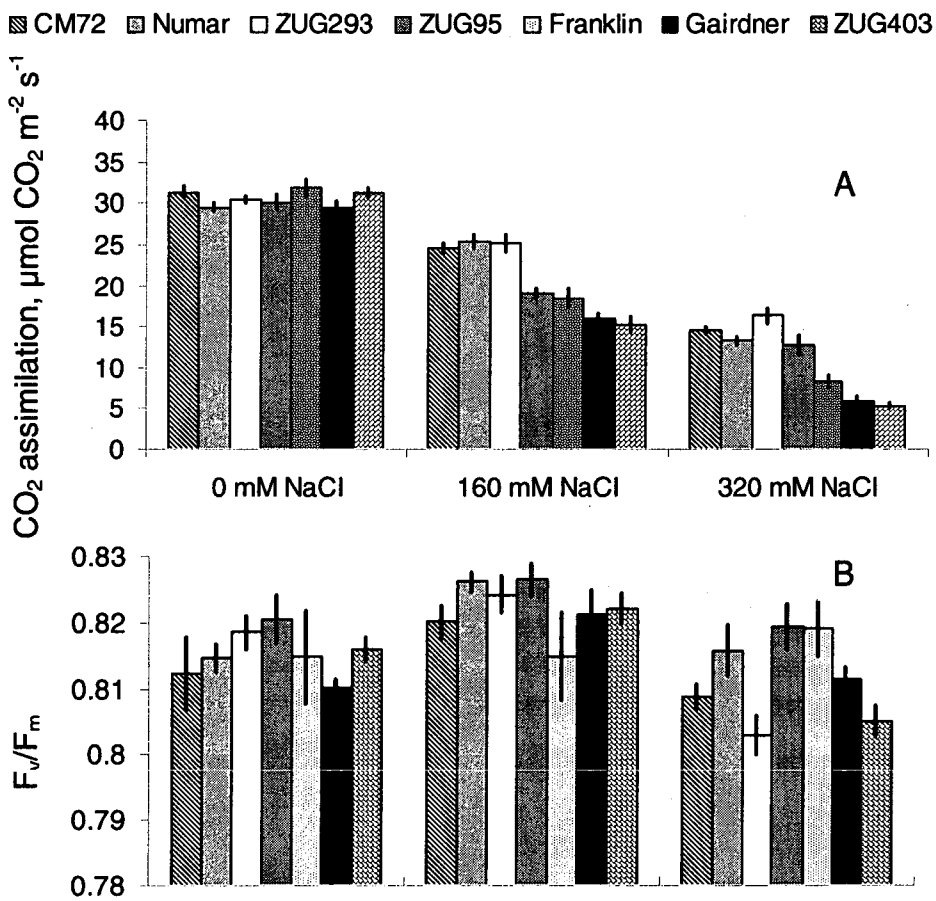

Figure 4.1. Effects of salinity on net CO_2 assimilation (P_n; A) and maximum photochemical efficiency of PSII (F_v/F_m value; B) of seven barley cultivars, measured after 5 weeks of three NaCl treatments. Means ± SE (n = 8).

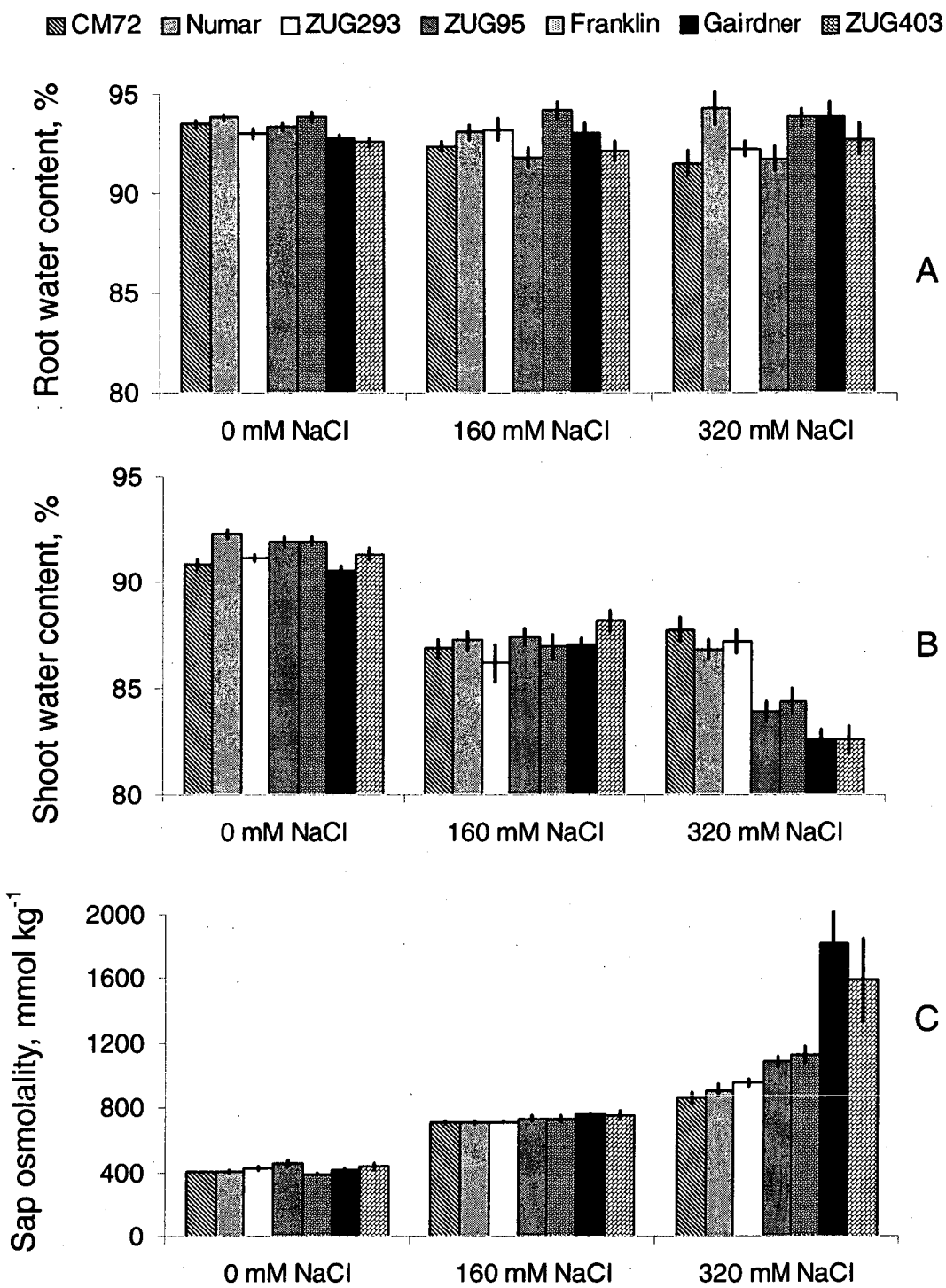

Figure 4.2. Effects of salinity on root (A) and shoot (B) water content and flag leaf sap osmolality (C) of seven barley cultivars after 5 weeks of exposure to three concentrations of NaCl. Means ± SE (n = 16 for A and B; n = 4 for C).

4.3.1.4. Na^+ and K^+ concentration in plant tissues

Consistent with the bulk of literature reports, salinity caused dramatic increases in both root and shoot Na^+ concentration as well as a significant ($P < 0.05$), several fold decrease in K^+ concentration in these tissues (Table 4.2). Interestingly, in roots the increase in Na^+ and decrease in K^+ was increasing with the severity of the salt stress (Table 4.2), while in shoots the difference between 160 mM and 320 mM treatment was only marginal and, in most cases, not significant. In terms of genotypic difference, the K^+ results appear to be much more sensitive indicators of salt tolerance than Na^+. As such, the difference in root Na^+ concentration between salt-tolerant (CM72 and Numar) and -sensitive (ZUG403 and Gairdner) cultivars were 20% for severe salt treatment and only 10% for moderate salt treatment. The corresponding proportions for root K^+ concentration were 5-fold and double (Table 4.2). The predictive value of K^+ measurements was much higher in root compared with leaf tissues.

Table 4.2. Root and shoot Na^+ and K^+ concentration and Na^+/K^+ ratio of seven barley cultivars after 5 weeks exposure to various NaCl levels. Means ± SE (n = 4).

Treatment	0 mM	160 mM	320 mM	0 mM	160 mM	320 mM
Cultivar	*Root Na^+ concentration, $\mu mol\ g^{-1}$ DW*			*Shoot Na^+ concentration, $\mu mol\ g^{-1}$ DW*		
CM72	80±3	1660±18	2830±85	40±4	2090±50	2720±17
Numar	80±4	1740±11	2860±46	40±7	2200±37	2880±11
ZUG293	80±4	1730±43	2880±35	40±5	2120±54	3040±22
ZUG95	90±6	1910±37	2790±57	50±6	2200±19	3210±45
Franklin	80±3	1970±26	3210±21	50±5	2720±12	3780±17
Gairdner	90±3	1920±35	3550±23	50±6	2560±22	3730±12
ZUG403	80±2	1890±17	3420±27	40±4	2550±47	3580±53
	Root K^+ concentration, $\mu mol\ g^{-1}$ DW			*Shoot K^+ concentration, $\mu mol\ g^{-1}$ DW*		
CM72	430±9	210±5	139±3	1000±14	430±3	450±8
Numar	400±6	250±3	110±1	970±12	420±7	420±5
ZUG293	360±3	230±3	130±3	930±11	450±4	440±8
ZUG95	360±4	170±1	84±5	920±6	390±5	420±8
Franklin	350±10	130±7	25±1	1050±10	280±2	340±7
Gairdner	350±10	70±4	25±2	1000±15	310±12	340±4
ZUG403	350±4	90±3	24±1	940±10	320±6	370±3
	Root Na^+/K^+ ratio			*Shoot Na^+/K^+ ratio*		
CM72	0.19±0.01	7.90±0.13	20.30±0.90	0.04±0.01	4.83±0.20	6.10±0.09
Numar	0.20±0.01	7.00±0.12	25.90±0.40	0.05±0.01	5.24±0.20	6.80±0.08
ZUG293	0.22±0.01	7.60±0.12	22.20±0.80	0.05±0.01	4.69±0.10	6.90±0.09
ZUG95	0.26±0.01	11.60±0.22	33.40±2.40	0.05±0.01	5.66±0.10	7.60±0.18
Franklin	0.24±0.01	15.80±0.46	129.00±1.40	0.04±0.01	9.96±0.50	11.20±0.17
Gairdner	0.25±0.01	27.90±1.76	146.00±7.80	0.05±0.01	8.42±0.30	10.90±0.12
ZUG403	0.24±0.01	21.90±0.54	144.00±5.90	0.04±0.01	7.97±0.20	9.70±0.15

4.3.2. Ion flux responses

4.3.2.1. NaCl-induced kinetics of Na^+, K^+, H^+ and Ca^{2+} flux

Salinity treatment caused immediate (within the time resolution of the MIFE system) changes in net Na^+, K^+, H^+ and Ca^{2+} fluxes from barley root surface (Figure 4.3). Regardless of the concentration used (20 to 160 mM), salinity treatment caused significant efflux of Ca^{2+} (Figure 4.3A). This NaCl-induced Ca^{2+} efflux was short-lived, not consistently related to tolerance, and not blocked by either Gd^{3+} or La^{3+}, two known Ca^{2+} channel blockers (data not shown). Therefore, the majority of the Ca^{2+} flux can be concluded as originating from the cell wall, as a result of the Donnan ion exchange, consistent with previous observations (Shabala and Newman 2000). It was not expected that the difference in salt tolerance between genotypes would be reflected in the Donnan exchange patterns in the root apoplast.

Salinity treatment also caused significant H^+ efflux from barley root surface (Figure 4.3B). Although this efflux was larger in salt-sensitive than in salt-tolerant cultivars (data not shown), no clear dose-response relationship was found (Figure 4.3B). Also, a multiphase (and often oscillatory) type of response made interpretation rather difficult.

Transient Na^+ flux responses (Figure 4.3C) were misleading, with at least two issues complicating their analysis. Firstly, Na^+ flux noise was much greater than for other cations, due to the high Na^+ background level (see the Na^+, K^+ and H^+ traces for 160 mM treatment; Figure 4.3). The second problem was non-ideal selectivity of the Na^+ liquid ion exchanger (LIX) used in this study. Methodological experiments showed that the Na^+ LIX was sensitive not only to Na^+, but also to K^+ and Ca^{2+} (Figure 4.4), with almost ideal Nernst response for each ion alone. Therefore, the apparent Na^+ efflux observed in response to NaCl treatment (Figure 4.3C) is expected to be an artefact, caused by the Na^+ LIX measuring NaCl-induced Ca^{2+} and K^+ efflux in addition to Na^+ influx. It is also obvious that net Na^+ influx measured in the first minute after NaCl treatment is greatly underestimated for the same reason.

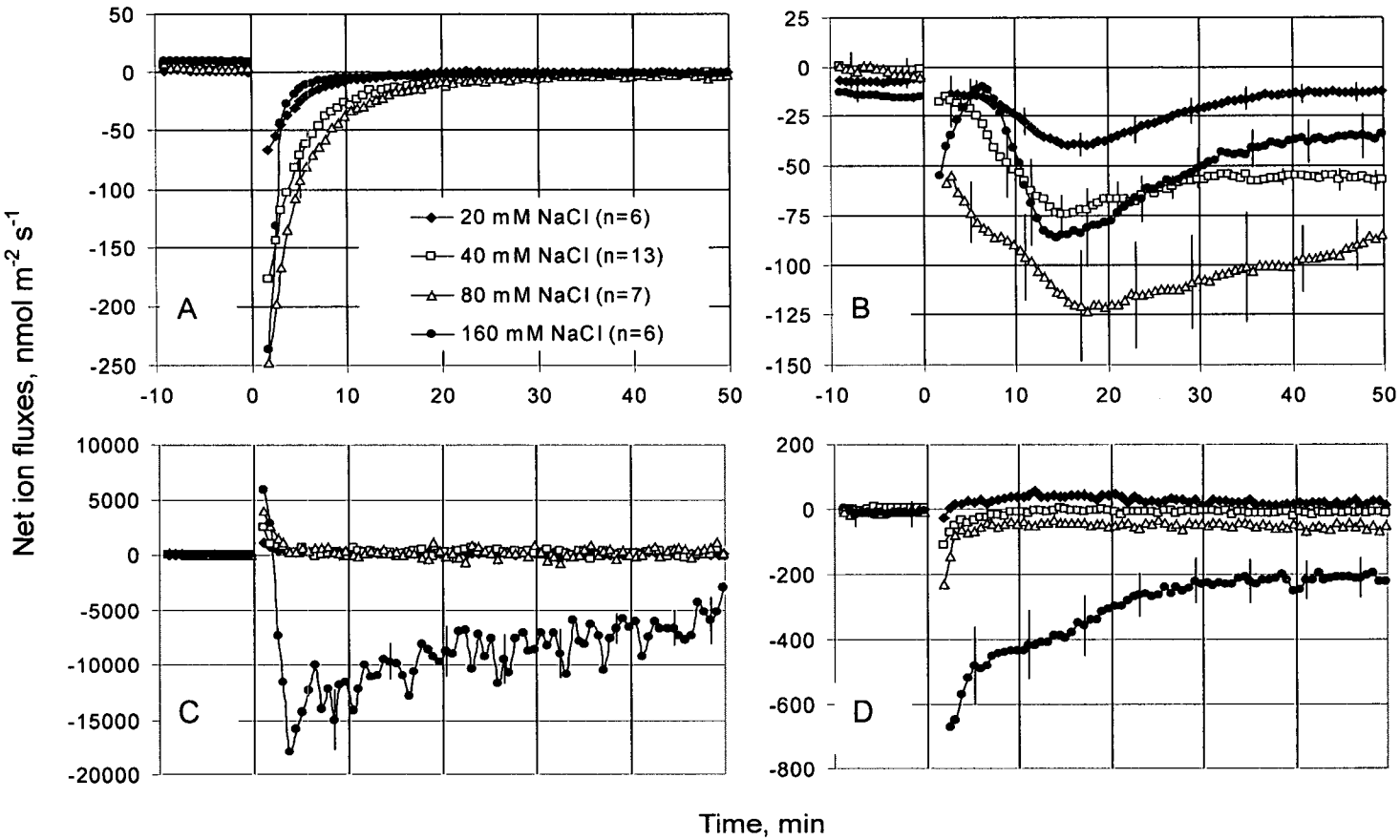

Figure 4.3. Transient net Ca^{2+} (A), H^+ (B), Na^+ (C) and K^+ (D) fluxes (influx positive), measured from mature zone (10 mm from root tip) of salt-sensitive Franklin in response to a range of NaCl treatments (applied at time 0 as indicated by arrows). Means ± SE (n = 6–13).

The most consistent results were found for K^+ flux. Salinity treatment caused immediate K^+ efflux, which gradually recovered over the next 30 to 40 min (Figure 4.3D). A clear dose-response relationship between the severity of salt stress and magnitude of K^+ efflux was found. Taken together with the relatively low level of K^+ flux noise, it was concluded that amongst the four ions measured in this study, K^+ flux measurements are the most convenient tool to discriminate between barley genotypes for salt tolerance. Accordingly, only K^+ flux was measured in all further studies.

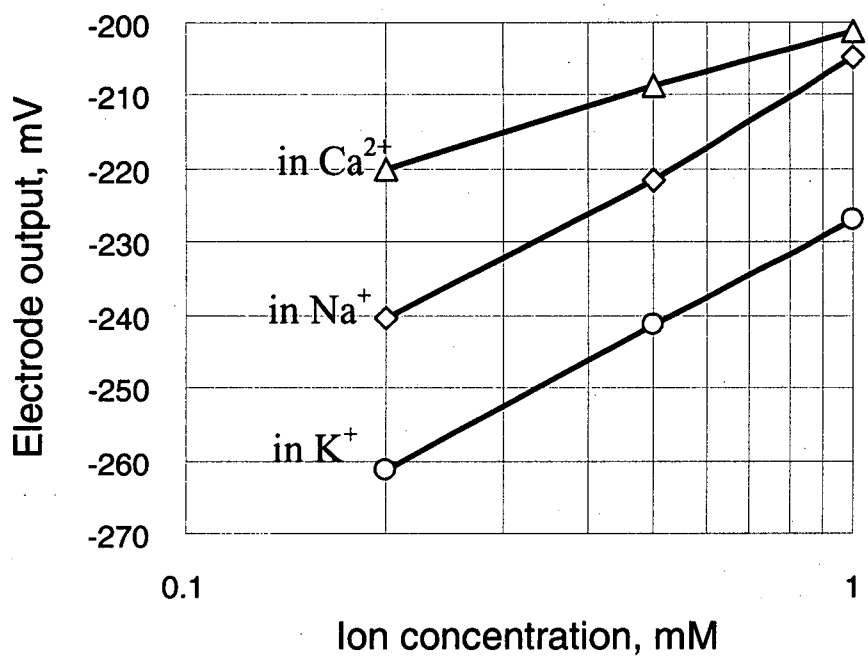

Figure 4.4. Non-ideal selectivity of Na^+ LIX. Na^+ electrode output is plotted *vs* concentration of the ion used for calibration. Na^+ electrodes were calibrated in three sets of solutions (NaCl, KCl, and $CaCl_2$, respectively), using 200, 500, and 1000 μM standards for each of them. One (out of 5) representative example is shown. Electrode characteristics were as follows: (1) Na^+ LIX in Na^+: slope, 50.72; intercept, -358.1; $r^2 = 0.998$. (2) Na^+ LIX in K^+: slope, 49.26; intercept, -374.9; $r^2 = 0.999$. (3) Na^+ LIX in Ca^{2+}: slope, 27.01; intercept, -282.9; $r^2 = 0.999$.

4.3.2.2. NaCl-induced K^+ flux profiles along the root axis

It was expected that functionally different root zones would show different K^+ flux responses. To test this hypothesis, transient K^+ flux kinetics was measured from different regions along the root axis (~ 0.6 mm increments) after 1 h exposure to 80

mM NaCl (Figure 4.5). These responses from the root apex were much stronger than from the mature root zone, with K^+ efflux of about 3500 nmol m^{-2} s^{-1} measured in the middle of the elongation zone (~ 3 mm from root tip). This is about 9-fold greater than average K^+ efflux in the mature zone (Figure 4.5). In contrast, no such sharp K^+ flux gradient was found in the control (open circles in Figure 4.5), although slightly higher K^+ uptake (~200 nmol m^{-2} s^{-1}) was observed in the root tip.

From Figure 4.5 it is clear that the sensitivity of K^+ flux measurements (signal to noise ratio) may be greatly enhanced when measurements are performed in the middle of the elongation zone. However, even a small (~ 0.5 mm) inaccuracy in electrode positioning might lead to a significant variation in the magnitude of K^+ efflux in response to salt treatment. Therefore, for comparison of different genotypes (with potentially different root anatomy), it is more prudent to compare K^+ flux data from the mature zone, where K^+ flux responses show less variability along the root length. Accordingly, all following measurements were conducted from the mature zone, ~ 10 mm from root tip.

4.3.2.3. Genotypic variation of NaCl-induced K^+ flux responses

Significant genotypic differences were found when net K^+ fluxes were measured from the mature root zone (~ 10 mm from root tip) in response to 80 mM NaCl treatment (Figure 4.6). Although all cultivars showed significant K^+ efflux in response to salinity, the magnitude of this efflux was significantly ($P < 0.001$) different between salt-tolerant and -sensitive genotypes. As such, average K^+ efflux over the interval 40 to 50 min after salt application was only 20–25 nmol m^{-2} s^{-1} for salt-tolerant CM72, Numar and ZUG 293, but 150–180 nmol m^{-2} s^{-1} for salt-sensitive Gairdner and ZUG403. Overall, a correlation between K^+ efflux and whole-plant responses was strong (Table 4.3), with most r^2 values being above 0.8. The highest correlation was found between K^+ efflux and shoot dry weight ($r^2 = 0.96$) followed by plant height ($r^2 = 0.94$) and osmolality of flag leaf ($r^2 = 0.91$) (Table 4.3).

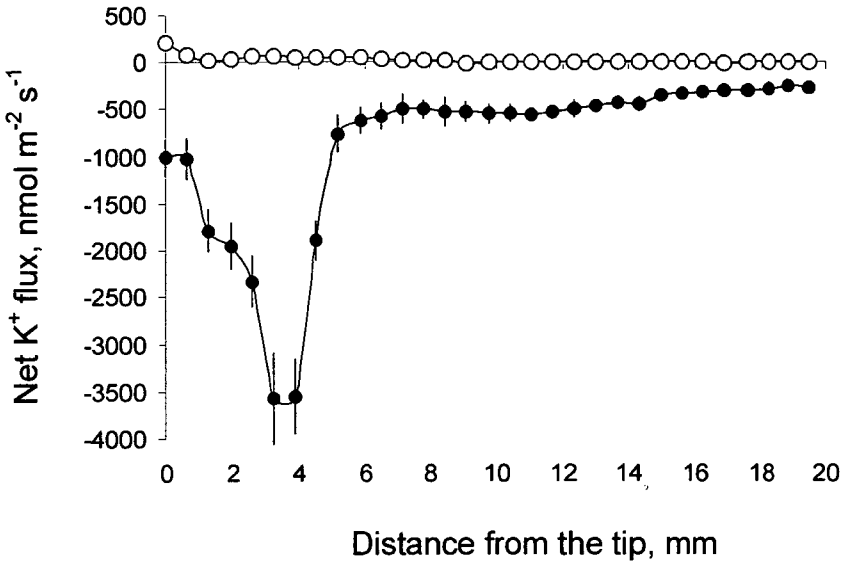

Figure 4.5. K^+ flux profiles along the root axis of salt-sensitive Franklin cultivar. Net K^+ fluxes were measured in control (open symbols) and after 1 h exposure to 80 mM NaCl (closed symbols) with 0.6 mm increments, starting from the root tip. At each position, an average K^+ flux was measured for 1–2 min before the electrode was repositioned. Means ± SE (n = 4).

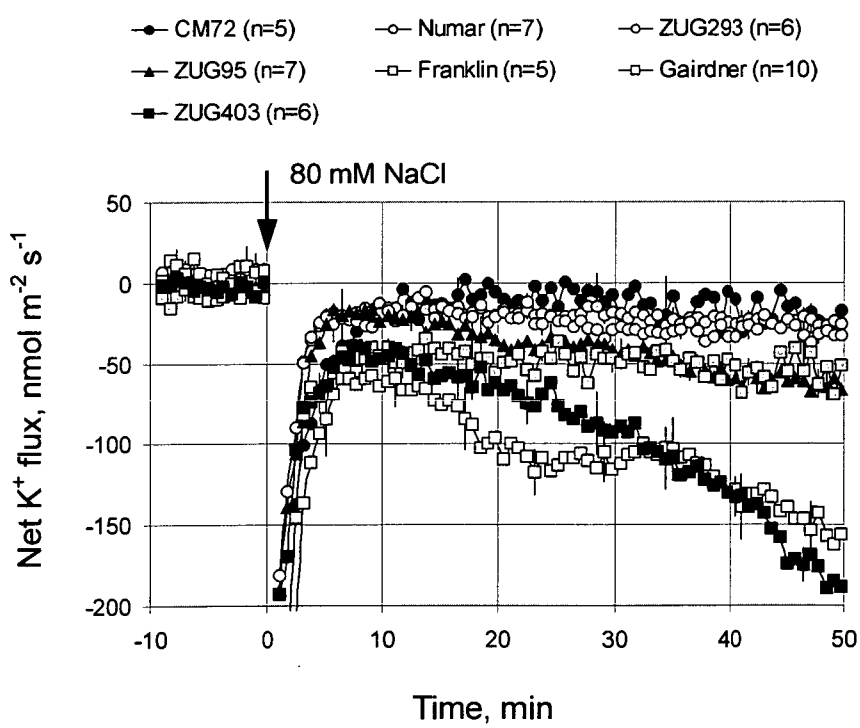

Figure 4.6. Net K^+ flux responses measured from seven barley cultivars following 80 mM NaCl treatment. Fluxes were measured in mature zone, about 10 mm from root tip. Means ± SE (n = 6–10).

Table 4.3. Linear correlation (r^2) between net K^+ efflux, measured from mature root zone 40 min after exposure to 80 mM NaCl, and changes in plant physiological characteristics of seven barley cultivars contrasting in salt tolerance.

Parameter	*Root*	*Shoot*
Fresh weight	0.59*	0.84**
Dry weight	0.79**	0.96***
Water content	0.32	0.80**
Na^+ concentration	0.76**	0.54
K^+ concentration	0.68*	0.32
Na^+/K^+ ratio	0.72*	0.5
Osmolality	0.91***	
Plant height	0.94***	
Net CO_2 assimilation	0.81**	

Note: significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.3.2.4. Specificity of NaCl-induced K^+ flux

In sharp contrast to 80 mM NaCl treatment, K^+ efflux in response to 20 mM NaCl was very short-lived (Figure 4.7A). A few minutes after NaCl application, a sustained net K^+ uptake was measured. No apparent genotypic difference was found for this treatment. A plausible hypothesis to explain such a contrast in response to very mild (20 mM) and more severe (80 mM) NaCl treatment was that in the former case, the osmotic component of the salt stress was dominating, while in the latter case, the specific ionic component had greater impact.

To test this hypothesis, barley roots of the salt-sensitive cultivar Gairdner were exposed to osmotic stress, using isotonic mannitol solutions (Figure 4.7B). Remarkably similar responses were found for 20 mM NaCl treatment and isotonic 35 mM mannitol treatment (Figure 4.7B). Qualitatively different responses were found, however, between 80 mM NaCl and isotonic 140 mM mannitol treatment (Figure 4.7B), validating the hypothesis.

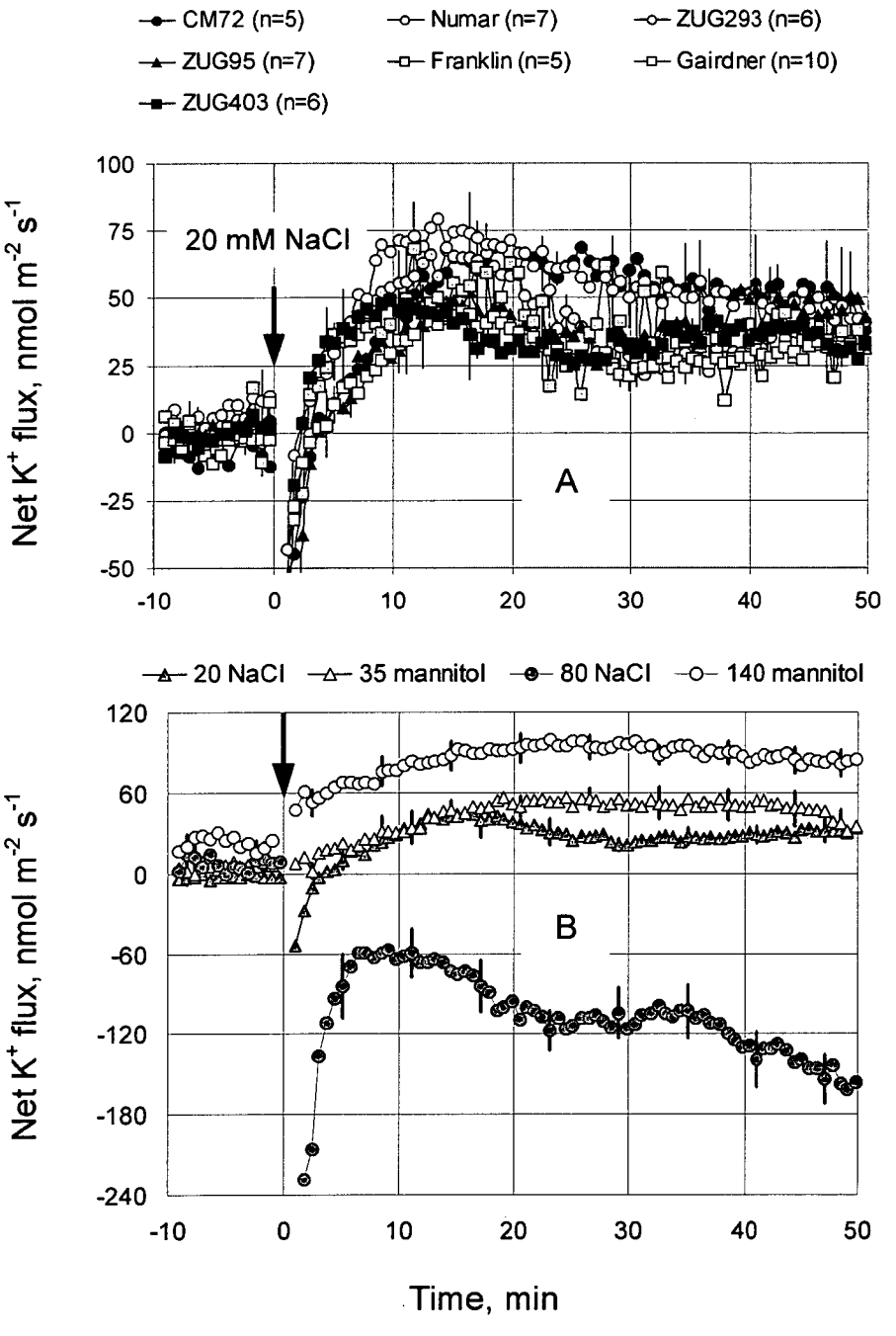

Figure 4.7. Specificity of salt-induced K^+ flux responses. (A) – net K^+ flux responses, measured from seven barley cultivars following 20 mM NaCl treatment. Fluxes were measured in mature zone, about 10 mm from root tip. Means ± SE (n = 6 to 10). (B) – net K^+ flux, measured from the mature zone of Gairdner, in response to isotonic NaCl and mannitol treatments. Means ± SE (n = 5–10).

4.3.3. Practical aspects of using K^+ efflux as a screening tool

4.3.3.1. Standardising conditions for K^+ flux measurement

In order to use K^+ flux measurements as a screening tool for salt tolerance, dose- and time- aspects of NaCl treatment should be optimised. Accordingly, all 7 cultivars used in this study were treated with a range of NaCl concentrations, from 20 to 160 mM (Figure 4.8A). Results showed that, although any concentration within the range 40 to 160 mM is suitable for screening, 80 mM treatment would be optimum, giving clear responses with good discrimination between barley cultivars for salt tolerance. The highest correlation ($r^2 = 0.93$) between K^+ efflux and salinity effects on whole plant biomass was found for this treatment (Table 4.4).

Table 4.4. Linear correlation (r^2) between net K^+ efflux and changes in whole plant biomass, measured from mature root zone for different times of exposure and severity of salt stress.

NaCl, mM 40 min exposure	r^2	*Time, min* 80 mM NaCl	r^2
20	0.41	40	**0.93****
40	0.85**	80	0.73*
80	**0.93****	140	0.53
160	0.84**	320	0.42

Note: significant at * $P < 0.05$; ** $P < 0.01$.

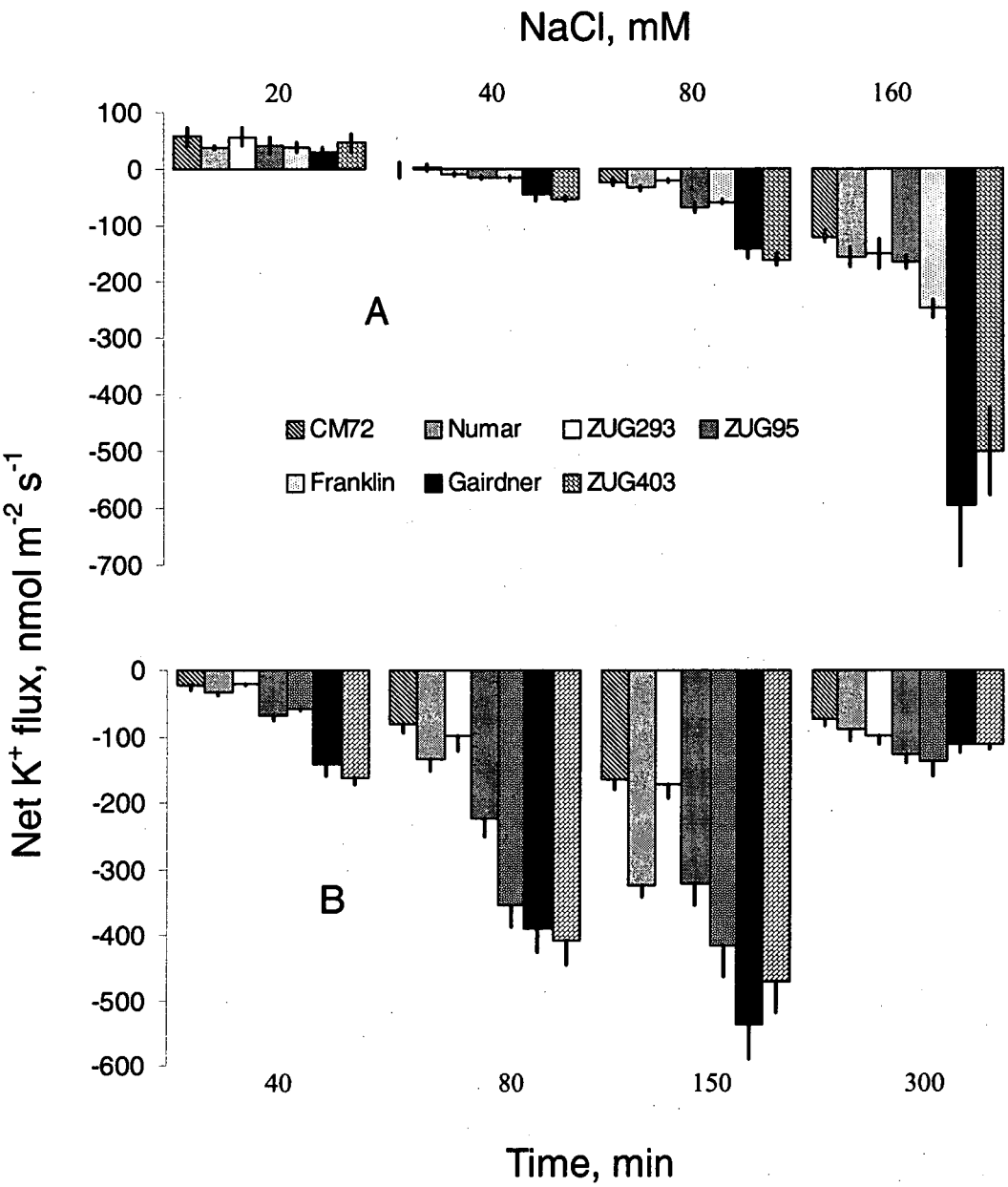

Figure 4.8. Dose (A) - and time (B) - dependence of K^+ flux responses. Net K^+ fluxes were measured from mature zone (10 mm from root tip) of seven barley cultivars. In panel A, mean K^+ fluxes (± SE; n = 6–10), measured 40 min after salt application, are plotted against four NaCl concentrations. In panel B, mean K^+ fluxes in response to 80 mM NaCl are plotted against the exposure time (averaged over the last 5 min). Means ± SE (n = 6–13).

Optimal timing was investigated by measuring root K^+ efflux after treatment for 40, 80, 150 and 300 min with 80 mM NaCl (Figure 4.8B). Correlation analysis (Table 4.4) showed that the r^2 value for 40 min treatment was the highest despite the 80 min treatment showing larger K^+ flux values (and, thus, better resolution). The 40-min treatment had also an advantage of being more rapid and thus more suitable for kinetics experiments.

The effect of seedling age on NaCl-induced K^+ flux responses was studied by using two contrasting (salt-tolerant CM72 and salt-sensitive Gairdner) cultivars. The two cultivars had similar root length at any given age (data not shown). The results showed that younger seedlings with shorter roots were more sensitive to salt stress (Figure 4.9), although genotypic differences were observed at any age.

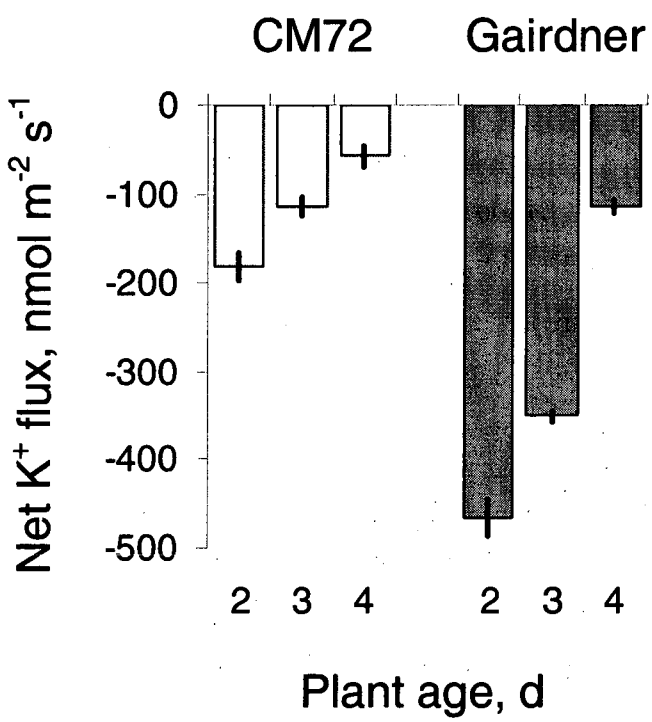

Figure 4.9. K^+ flux as a function of seedling age. Net K^+ flux was measured 40 min after root exposure to 80 mM NaCl from salt-tolerant CM72 and salt-sensitive Gairdner cultivars for seedlings of different age. Means ± SE (n = 6).

4.3.3.2. Testing applicability of K^+ flux for screening

Further validation of K^+ flux measurements as a screening tool for salt tolerance in barley was performed on crosses between salt-tolerant (CM72 and Numar; defined as T1 and T2, respectively) and -sensitive (Gairdner and Franklin; defined as S1 and S2, respectively) barley cultivars. As shown in Figure 4.10, the lowest K^+ efflux was measured from the two salt-tolerant cultivars and the cross between them (T1, T2 and T1T2), with net K^+ efflux being < 150 nmol m^{-2} s^{-1}. Highest K^+ efflux was observed from salt-sensitive S1 and S2 cultivars and the cross between them (S1S2), with net K^+ efflux exceeding 300 nmol m^{-2} s^{-1}. Crosses between salt-tolerant and -sensitive cultivars showed intermediate K^+ efflux (300 to 150 nmol m^{-2} s^{-1} ranges). The intermediate responses of the F_1s suggest additive genetic control of salinity tolerance. F_2s would need to be screened to study further inheritance of this trait (Chapter 6).

4.3.3.3. K^+ leakage into solution: an alternative technique for screening

As an alternative to the technically demanding MIFE flux measurements, K^+ net efflux from salinised roots could be measured by simpler means for quick routine screening in plant breeding. Accordingly, roots of uniform intact seedlings of each of four cultivars were immersed in 80 mM NaCl for 2 h. The leaked K^+ was measured by flame photometry. As shown in Figure 4.11, leakage of K^+ from the two salt-susceptible cultivars was, on average, 1.5-fold higher than from salt-tolerant cultivars (when expressed on a fresh weight basis). The difference between salt-tolerant and -sensitive genotypes is significant at $P < 0.05$.

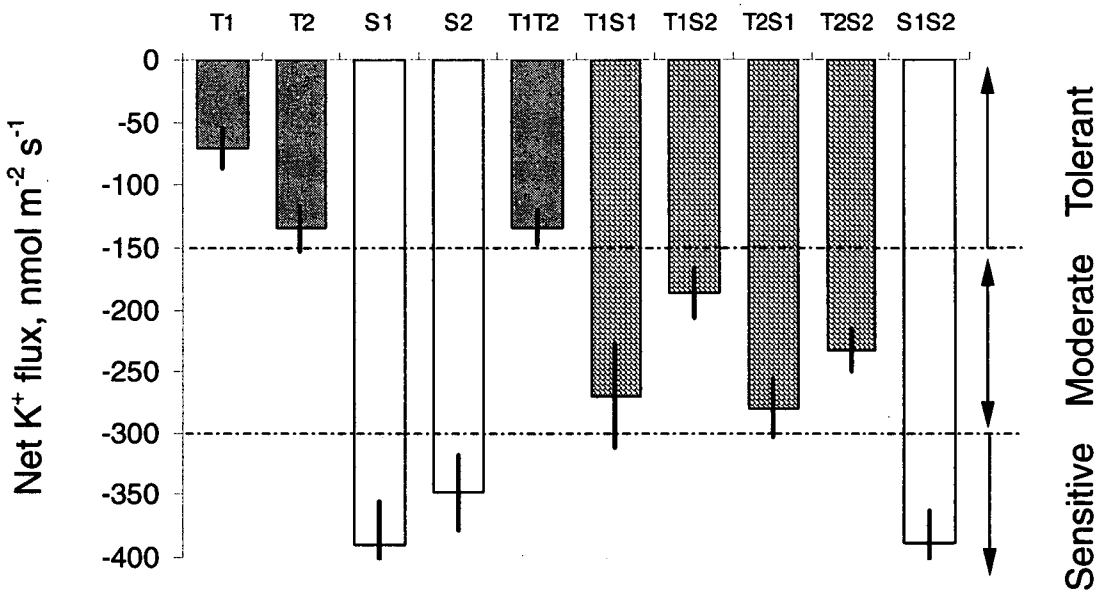

Figure 4.10. Net K^+ flux, measured from mature zone of roots of four barley cultivars of contrasting salt tolerance (T1, CM72; T2, Numar; S1, Gairdner; S2, Franklin) and their F_1 hybrid lines after 1 h pre-treatment in 80 mM NaCl. Means ± SE (n = 5–8).

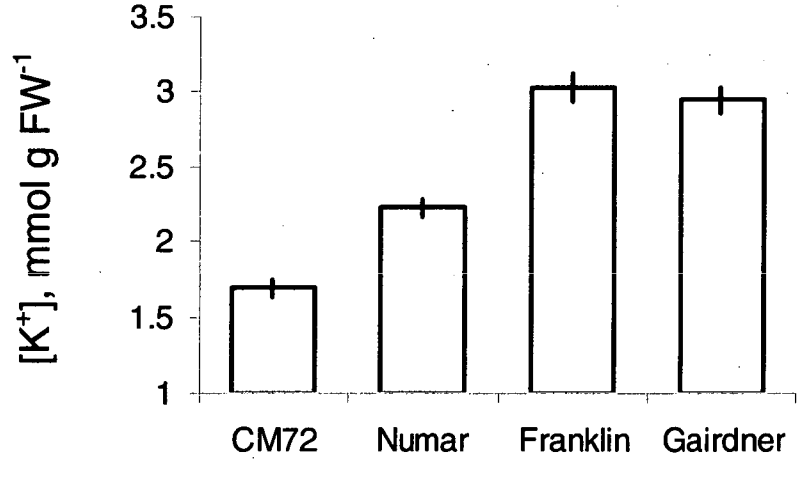

Figure 4.11. K^+ leakage from roots of four barley cultivars exposed to 80 mM salinity for 2 h. The amount of K^+ released into solution was measured by flame photometer and calculated per root FW. Means ± SE (n = 10).

4.4. Discussion

4.4.1. Physiological mechanisms of salt tolerance in barley

4.4.1.1. Genetic diversity of barley

The magnitude and distribution of genetic diversity in wild plants is a major factor of continuous natural evolutionary processes (Graner et al. 2003). In nature, barley is present over a wide range of habitats. It is found widely scattered over most temperate parts of the world and is well represented in the world's gene banks with ca 378,000 accessions reported (Hintum and Menting 2003). In addition to nature, the same basic evolutionary processes of mutation, recombination and selection operating under domestication and breeding have changed barley considerably under cultivation, especially in the past 100 years (Bothmer et al. 2003). Such wide genetic diversity makes it possible to identify some salt-tolerant genotypes and use them in breeding programmes to combine stress tolerance with high yield. Earlier, Pakniyat et al. (1997) found a very strong correlation between habitat and stress tolerance such as salt and drought in a range of Israeli barley. In the experiments reported here, three cultivars, identified as salt-tolerant, were all originally from arid or semiarid areas. The genetic diversity of barley also enabled us to select a range of cultivars to investigate mechanisms underlying salt tolerance and to develop a simple screening criterion for this trait.

4.4.1.2. Growth limitation: stomatal *vs* non-stomatal inhibition of photosynthesis

At the cellular level, growth rate reduction under salt stress results from inhibition of both cell division and expansion (Zhu 2001b) and is an adaptive feature for plant survival. At the whole-plant level, reduced rate of growth (and, ultimately, biomass) is due to impaired photosynthetic performance under saline conditions. The latter may be a result of either response of stomatal conductance to salinisation (so-called stomatal limitation of photosynthesis) or limited capacity of the plant for CO_2 fixation, independent of diffusion limitations (non-stomatal limitation of photosynthesis) (Seemann and Critchley 1985; Zhu 2001b; Munns 2002). It remained to be discovered

which of these components dominates in barley.

It is generally accepted that, at intermediate salinities, the predominant mechanism is the stomatal limitation of photosynthesis, and that the nonstomatal limitation at the biochemical level prevails under more severe salinity (Seemann and Critchley 1985; Plaut et al. 1989; Bethke and Drew 1992; Everard et al. 1994). However, my results (Figure 4.1B) showed no apparent effect of salt stress on F_v/F_m. Regardless of severity of the salt stress, the F_v/F_m values remained above 0.8, indicating optimal functioning of PSII. Thus, although F_v/F_m values have been widely used as a non-destructive and non-invasive tool to determine effects of environmental stresses on the photosynthetic apparatus (Maxwell and Johnson 2000; Shabala 2002; Sayed 2003), it is not likely that these measurements are appropriate to screen barley for salt tolerance. It appears that even under severe stress conditions leaf photochemistry in barley is well protected. This conclusion is consistent with previous observations on maize (Shabala et al. 1998).

Despite the absence of any apparent effects of salinity on leaf photochemistry, NaCl treatment caused a very significant reduction in P_n (Figure 4.1A), the effect being closely correlated with biomass and plant height ($r^2 = 0.85$ and 0.79 respectively, $P < 0.01$). Therefore, it appears that stomatal limitation of photosynthesis is a major component reducing barley growth in saline conditions.

Consistent with these results were RWC (Figure 4.2). Salt tolerance is not exclusively correlated with adaptation to toxicity of Na^+ but also reflects adaptation to secondary effects of salinity such as water deficit and impaired nutrient acquisition (Flowers et al. 1977; Greenway and Munns 1980). In experiments reported here, root water content was not significantly affected (Figure 4.2A), indicating efficient osmotic adjustment in barley roots. SWC, however, was significantly reduced by salinity (Figure 4.2B), with the effect increasing with the severity of salt stress and showing high ($r^2 = 0.97$, $P < 0.001$) correlation with salt tolerance.

It is interesting to notice an approximately 2-fold difference in the osmolality of the flag leaf sap between salt-tolerant and -sensitive barley cultivars (Figure 4.2C) for the 320 mM NaCl treatment. At the same time, only 30% difference in leaf Na^+ was found for these treatments (Table 4.2). Therefore, it is not likely that higher Na^+ accumulation in the mesophyll cells of salt-sensitive cultivars is responsible for such a sharp difference in leaf osmolality. It might be interesting in future work to measure the elemental composition of other major inorganic ions and organic osmolytes

potentially contributing to osmotic adjustment of salinised barley leaves (Chapter 8).

4.4.2. Root ion fluxes and salt tolerance in barley

4.4.2.1. K^+/Na^+ relations in salinised tissues

The capacity of plants to counteract salinity stress strongly depends on the status of their K^+ nutrition (Maathuis and Amtmann 1999). Similarity in physical and chemical structure of Na^+ and K^+ results in strong impairment of a large number of K^+-dependent metabolic processes by elevated Na^+ in the cytosol. In addition, because many K^+ transport systems have some affinity for Na^+ transport (Schachtman and Liu 1999; Blumwald et al. 2000; Véry and Sentenac 2002; Shabala 2003), elevated external Na^+ disrupts K^+ homeostasis in the cell by competing with K^+ for the same uptake sites.

In this study, salt stress significantly lowered K^+ concentration in both roots and shoots of all the cultivars studied (Table 4.2), but the inhibitory effects of NaCl were much less in the salt-tolerant cultivars than in the salt-susceptible ones. As the tissue which directly confronts the salinity stress, roots exhibited more sensitivity than the shoots. Thus, 320 mM NaCl-induced stress caused a 14-fold decrease in root K^+ concentration in salt-sensitive Franklin, Gairdner and ZUG403 cultivars, but only 3-fold decrease in salt-tolerant CM72 and Numar cultivars (Table 4.2). At the same time, the difference in root Na^+ concentration was less than 40%. This suggested that the ability of plant cells to retain K^+ is crucial for salt tolerance, rather than their ability to restrict Na^+ from uptake, at least in barley. As a result of a better ability to retain K^+, salt-tolerant cultivars were able to maintain higher K^+/Na^+ in the root, enabling better performance in saline conditions. This is consistent with literature reports (Santa-Cruz et al. 1999; Zhu et al. 1998; Nublat et al. 2001; Tester and Davenport 2003).

4.4.2.2. K^+ and root osmotic adjustment

Under saline conditions, plant cells must readjust their osmotic potential to prevent water loss. The latter is achieved either by enhanced uptake of inorganic ions or by *de novo* synthesis of organic osmolytes (Serrano et al. 1999a; Shabala and Lew 2002).

These responses appear to be stress-specific. Consistent with previous reports on *Arabidopsis* roots (Shabala and Lew 2002) and bean leaf mesophyll (Shabala et al. 2000), hyperosmotic treatment with mannitol caused rapid and sustained K^+ uptake into barley roots (Figure 4.7B), with the magnitude of K^+ influx increasing with severity of osmotic stress. Interestingly, responses to 20 mM NaCl (Figure 4.7A) were very similar to those for isotonic 35 mM mannitol solution (Figure 4.7B). This suggests that, under mild salinity conditions, the osmotic component of salt stress was dominating, and barley roots took up inorganic cations (specifically, K^+) instead of following the energy-expensive (Raven 1985) avenue of *de novo* synthesis of compatible solutes. This is in accord with earlier literature reports (Cerda et al. 1995; Huang and Redmann 1995).

When roots were exposed to higher NaCl levels, net K^+ efflux was measured (Figure 4.3D and 4.8). This efflux is likely to be mediated by depolarisation-activated K^+ channels as suggested from membrane potential measurements (Shabala et al. 2003) and patch-clamp studies (Shabala et al. 2006a) on barley and *Arabidopsis* roots. At the same time, isotonic 140 mM mannitol treatment caused a significant and sustained K^+ uptake (Figure 4.7B). Therefore, it appears that K^+ fluxes from root epidermis under salt conditions are driven by two oppositely directed signals: (1) K^+ efflux resulting from NaCl-induced PM depolarisation and (2) K^+ uptake resulting from some elusive "osmosensing mechanism". Under mild salinities, the latter component is dominating, while higher NaCl treatments result in overall net K^+ efflux from salinised roots.

4.4.2.3. Use of non-invasive ion-selective microelectrodes to screen barley for salt tolerance

The most obvious and logical way to screen plants for salt tolerance would be measuring net Na^+ fluxes from the root surface. The ability of roots to exclude Na^+ from uptake, via either restricting Na^+ influx or via enhanced Na^+ extrusion from the cytosol (Tyerman and Skerrett 1999; Blumwald 2000; Tester and Davenport 2003), has always been considered as a key feature of salt tolerance. Various techniques have been employed, ranging from patch-clamp studies on Na^+ currents through Na^+-permeable channels (Roberts and Tester 1997) to measuring whole root $^{22}Na^+$ influx by radiotracers (Essah et al. 2003). Unfortunately, none of these techniques

appears to be suitable as a screening tool. Can Na^+ selective microelectrodes be used instead?

Results reported here question the applicability of Na^+ selective microelectrodes for plant screening and suggest that, in most cases, measured Na^+ flux was merely an artefact. The latter occurs from non-ideal selectivity of all commercially available Na^+ LIX. It is well known that poor discrimination between Na^+ and K^+ of the microelectrode LIX molecule severely limits the range of Na^+ measurements (Carden et al. 2001). Recently an analytical procedure has been suggested, which allows accurate determination of ionic concentrations in the presence of one interfering ion, whence ion-fluxes may be routinely calculated (Knowles and Shabala 2005). However, it appears that, in addition to being K^+ sensitive, Na^+ LIX is also highly sensitive to Ca^{2+} (Figure 4.4). In response to acute salt stress, extruding K^+ and H^+ ions (Figure 4.4) will replace a significant part of cell wall Ca^{2+} from the Donnan space. This Ca^{2+} efflux will be measured and interpreted by Na^+ LIX as net Na^+ efflux (Figure 4.3C). It is known that a microelectrode with a non-ideally selective membrane responds both to the ion of interest and other ions, known as interfering ions, and there is not a simple relation between the electrical potential measured by the electrode and the external ionic activity (Ammann 1986). As a result, separation of the "real" Na^+ flux from artefacts caused by non-ideal Na^+ LIX selectivity is extremely challenging task (especially in the case of two interfering ions – K^+ and Ca^{2+}), and I am currently not aware of any analytical procedure applicable to solve this problem. Last but not least, at high concentrations of an ion, the voltage change for a given flux becomes smaller in proportion, resulting in relatively low sensitivity of Na^+ LIX at high external NaCl concentration. According to Ryan et al. (1990), the minimum detectable Na^+ flux at 80 mM NaCl is ~1300 nmol m^{-2} s^{-1}. All this makes the use of Na^+ selective microelectrodes in salinity studies very problematic.

The applicability of measuring fluxes of the other two ions (H^+ and Ca^{2+}) for screening purposes is also questionable. In general, H^+ efflux followed dose-response patterns in the NaCl range 20 to 80 mM (Figure 4.3B), with relative consistency between the salt-tolerant and -sensitive barley observed after 20 min of treatment (data not shown). However, addition of 160 mM NaCl caused surprisingly small H^+ efflux (even smaller than in roots treated with 40 mM NaCl), with some seedlings even showing H^+ uptake in the first 10 min (data not shown). It is likely that numerous H^+ transport systems are affected by salinity, both directly and indirectly,

and a direct causal link between salinity stress and measured net H^+ flux is difficult to establish. As for NaCl-induced Ca^{2+} efflux, most of it originated from the cell wall (see Shabala and Newman 2000, for details). It is hardly surprising, therefore, that no clear dose-response dependence was found (Figure 4.3A), and no correlation between the magnitude of Ca^{2+} efflux and cultivar salt tolerance was found (data not shown).

In sharp contrast to the above, K^+ flux measurements showed strong correlations with plant salt tolerance (Figures 4.6 and 4.8; Table 4.3) and therefore provide suitable discrimination for salinity tolerance between barley cultivars. As evident from Figure 6, average K^+ efflux over the 40 to 50 min interval after salt application was only 20–25 nmol m^{-2} s^{-1} for salt-tolerant CM72, Numar and ZUG 293 but 150–180 nmol m^{-2} s^{-1} for salt-sensitive Gairdner and ZUG403. This 6-fold K^+ efflux ratio between salt-tolerant and -susceptible barley showed a wider genotypic dispersion than other physiological parameters measured (root Na^+ concentration, 1.2-fold; flag leaf sap osmolality, 1.7-fold; plant biomass, 2.2-fold; P_n, 2.3-fold). It was very similar to root K^+ concentration (5.2-fold) and K^+/Na^+ ratio (6.2-fold). Thus, it appears that root K^+ efflux in response to NaCl treatment may be used as a reliable, non-destructive (although indirect) measure of the intracellular K^+/Na^+ ratio, which is crucial for plant salt tolerance (Maathuis and Amtmann 1999).

In this study, variation in K^+ efflux in the mature root zone was observed to be additive, with the F_1 being intermediate between the salt-tolerant and -sensitive parents (Figure 4.10). F_2 or backcross data would be needed to calculate heritability, but the data are in accord with findings of García et al. (1997) that the overall K^+/Na^+ ratio was heritable in wheat, and further supports the use of K^+ efflux as a reliable indicator of salt tolerance in barley. As far as physiological variability of other ions was concerned, K^+ efflux was comparatively less variable within a certain cultivar regardless of NaCl concentrations or duration of pre-treatments.

4.4.3. K^+ efflux as a screening tool: practicalities

4.4.3.1. Mature root zone gives optimum flux measurement

There are some reports discussing the relationship between root maturation and ion uptake patterns. Ryan et al. (1990) and Piñeros et al. (1998) suggested that essential

(K^+ and Ca^{2+}) and toxic (Cd^{2+}) cations showed variable fluxes from the root tip to the mature zone. Moreover, the presence or absence of suberin may be responsible for the spatial changes of K^+ flux along the root (Ryan et al. 1990). Differential sensitivity of ion transporters in mature and elongation root zones was reported in response to hormonal treatment (Ludidi et al. 2004), cadmium (Piñeros et al. 1998) and aluminium stress (Huang et al. 1993). In this study, a 9-fold difference between NaCl-induced K^+ efflux from elongation and mature zone of barley roots was reported (Figure 4.5). Although higher K^+ flux response might be advantageous in terms of the signal to noise resolution, the sharp gradient in K^+ flux responses within the root apex poses a serious methodological problem. Even a small mistake in measurement positioning or genetic variation in root anatomy among cultivars will lead to a huge error in estimating K^+ flux (Figure 4.5). From this point of view, more uniform K^+ flux responses in the mature zone in both control and treated plants are likely to be much more reliable for practical purposes of plant screening.

4.4.3.2. Standardising the NaCl concentration, treatment time and root age

There was a clear correlation between plant age and the magnitude of NaCl-induced K^+ efflux, with younger roots being more sensitive (Figure 4.9). It remains to be answered whether these changes are attributable to changes in root anatomy (e.g. extent of root suberisation), or whether they reflect altering sensitivity of PM transporters. For practical purposes of screening, "the younger the better" can be used as a guide, provided that the seedling can be easily handled and immobilised in the measuring chamber. As for optimal concentration and timing of treatment, the highest correlation between K^+ efflux and plant salt tolerance was found to be for 80 mM NaCl treatment and 40 min exposure (Table 4.4), although consistent results were observed in a wide range of times and concentrations (Figure 4.8). Therefore, for practical purposes of screening, I suggest that 3-d old roots treated in 80 mM NaCl for 40 min should be used to reliably discriminate for salt tolerance of barley. However, as barley is one of the most salt-tolerant glycophytes, salt concentrations should be carefully tested in dealing with the diversity of salt tolerance in other species.

4.4.3.3. Practical aspects

As shown and discussed above, non-invasive K^+ flux measurements provide a reliable

non-destructive estimate of salt tolerance in barley. However, the MIFE technique *per se*, as well as its analogues (e.g. Smith 1995), is rather skill demanding. Even in experienced hands, less than 10 plants per day can be measured when studying transient flux responses of 40 min duration. Although reliable, this may not be sufficient for a screening tool. The efficacy of the method can be significantly (at least one order) increased if roots are pretreated for a certain amount of time (e.g. 40 min to 2 h) in NaCl solution, and steady state fluxes are then measured for only a relatively short (1-2 min) period. The bottleneck of such a protocol would be standardizing the time of treatment (Figure 4.8).

As a viable practical alternative to non-invasive K^+ flux measurements, another screening technique was developed based on the amount of K^+ leaked from roots after exposure to NaCl. Being very simple, without micromanipulation, this technique does not require any sophisticated equipment. The results suggested that such a technique can relatively accurately quantify K^+ efflux from the root, and is sensitive enough to discriminate between salt-sensitive and -tolerant cultivars (Figure 4.11). Standing on the same theoretical foundation, such a method makes it possible to screen thousands of seedlings per day and could be used, therefore, by plant breeders for practical screening purposes for salt tolerance.

Chapter 5. Potassium and Sodium Relations in Salinised Barley Tissues as a Basis of Differential Salt Tolerance

5.1. Introduction

High Na^+ tissue concentration has often been considered as the major factor responsible for salt toxicity in non-halophytes. It is conventionally assumed that an ability to exclude Na^+ correlates with plant salt tolerance (Munns and James 2003; Garthwaite et al. 2005). However, no significant difference in unidirectional Na^+ uptake (measured as $^{22}Na^+$ influx) was found between wheat cultivars contrasting in their salt tolerance (Davenport et al. 1997). Although a correlation between grain yield and Na^+ exclusion from leaves has been shown in wheat (Chhipa and Lal 1995; Ashraf and Khanum 1997), it was not observed across all genotypes (El-Hendawy et al. 2005), suggesting that Na^+ exclusion is not the only mechanism determining salt tolerance in this species. It was also shown that bread wheat (hexaploid, ABD genomes) had not only a low rate of Na^+ accumulation, but also an enhanced K^+/Na^+ discrimination, a feature controlled by a *Kna1* locus on chromosome 4D (Dvořák and Gorham 1992; Dvořák et al. 1994; Dubcovsky et al. 1996). Importantly, this locus was not present in durum wheat (tetraploid, AB genomes), which is considered to be more salt-sensitive than bread wheat (Gorham et al. 1987, 1990; Munns et al. 2000). Taken together, these reports indicate that a plant's capacity to maintain a high cytosolic K^+/Na^+ ratio is a crucial determinant of salt tolerance.

The importance of maintaining an optimal K^+/Na^+ ratio for plant salt tolerance is hardly surprising and is well discussed in the literature (Gorham et al. 1991; Gaxiola et al. 1992; Cuin et al. 2003). It is also obvious that such an optimal ratio can be maintained by either restricting Na^+ accumulation in plant tissues or by preventing K^+ loss from the cell. Surprisingly, it is the former mechanism that has been in the spotlight of plant breeders (Heenan et al. 1988; Ashraf and Khanum 1997; Garthwaite et al. 2005). To the best of my knowledge, no large-scale screening for plant salt tolerance has been undertaken based on measuring the ability of plants to retain K^+ in their tissues.

The importance of the latter ability is further emphasised by the recent studies of

Arabidopsis sos mutants that highlighted the importance of potassium homeostasis for salt tolerance (Liu and Zhu 1998; Zhu et al. 1998; Rus et al. 2004; Shabala et al. 2005a). Electrophysiological analysis of root cation channels showed that all major K^+ uptake systems exhibit higher K^+/Na^+ selectivity in salt cress (salt-tolerant relative) than in *Arabidopsis* (Volkov et al. 2004). Other papers have also suggested that a better selectivity of cation transport systems for K^+ over Na^+ is an important salt tolerance determinant (Rodríguez-Navarro 2000).

In Chapter 4, a strong correlation was reported between the ability of barley roots to restrict NaCl-induced K^+ efflux and the plant's salt tolerance (Chen et al. 2005). The results showed that measuring net K^+ flux from roots of 3-d old seedlings in response to NaCl treatment provided suitable discrimination for salinity tolerance in barley. However, the above results were based on analysis of only seven barley cultivars. For such a polygenic trait as salt tolerance, this sample size is clearly not large enough to convince a plant breeder that this trait can indeed be used as a key determinant in breeding plants for salt tolerance. Accordingly, the aim of this work was to validate the applicability of K^+ flux measurements from salinised roots as a selection criterion for breeding barley for salt tolerance. This validation was done by conducting two large-scale glasshouse trials, including nearly 70 barley cultivars (~ 5300 plants in total), and comparing their physiological and yield performance with non-invasive microelectrode measurements of NaCl-induced K^+ flux from roots of 3-d old seedlings measured in laboratory conditions. Overall, the results showed that K^+ flux from the root surface in response to NaCl treatment was highly correlated ($P < 0.001$) with major plant physiological characteristics (relative grain yield, shoot biomass, plant height, net CO_2 assimilation, survival rate, and seed weight measured in glasshouse experiments after 4 to 6 months of salinity treatment). This emphasises the critical role of K^+ homeostasis in plant salt tolerance in barley and reinforces the applicability of using K^+ flux measurement as a reliable screening tool in barley breeding programmes.

5.2. Materials and methods

5.2.1. Plant materials and growth conditions

Two experiments were conducted, in 2004 (November to February; referred as Trial 1) and 2005 (June to December; Trial 2), with 62 and 69 barley genotypes studied, respectively. Overall average temperatures were 22.7 and 19.6°C, and average humidity 56.3 and 68.0%, respectively in Trial 1 and Trial 2. Control (standard potting mix) and salt treatment (NaCl added to potting mix to result in approximately 30 dS m^{-1} conductivity of saturated paste extract) were conducted with a completely randomised design with four replicates. All the plants were carefully hand-watered during the experiments, with any water in the saucers returned to the pots to avoid any potential salt leaching.

5.2.2. Growth and yield components

The emerged seedlings (referred to as germination elsewhere) were observed daily in the first 3 weeks, and number of surviving plants and their height immediately prior to harvesting were recorded. Plants were harvested at about four months of age in Trial 1 (summer) and six months of age in Trial 2 (winter-spring). A collective sample of the shoot biomass and grain yield for all plants in each pot (five in total) was taken. The number of tillers on each plant was counted. Mean seed weight was calculated from the number of seeds and their total weight.

5.2.3. CO_2 assimilation

P_n was measured from flag leaves by an LCi portable infrared gas analyser (ADC BioScientific, Hoddesdon, UK) in Trial 1 at the age of two months shortly before the plants started heading. More details are in 3.4.4.

5.2.4. K^+ flux measurement

Details of K^+ flux measurement are in the General Materials and Methods 3.3.2.

5.3. Results

5.3.1. Effect of salinity on various physiological characteristics

Nearly 70 genotypes were tested over the two consecutive seasons with a total of ~ 5300 seedlings. Much higher shoot biomass and grain yield were observed in both control and treated plants for Trial 2 than Trial 1, which was primarily a result of the different duration of growth (six and four months, respectively), as well as growth conditions (winter-spring *vs* summer). Despite some differences in absolute numbers, the overall performance of individual cultivars was mostly consistent for the two seasons (Table 5.1). Salinity treatment significantly ($P < 0.01$) reduced grain yield, shoot biomass, and thousand seed weight (TSW) of barley plants (Table 5.1 and Figure 5.1A, B and G). The impact of salinity differed substantially between barley cultivars, with salt-sensitive genotypes (such as Yangsimai 3, Gairdner, and Yan 96219) giving zero grain yield under saline conditions, while salt-tolerant genotypes (such as Numar, ZUG293 and CM72) yielded 20 to 25% of their control values (Table 5.1). This reduction in grain yield and shoot biomass was most likely a consequence of reduced P_n under saline conditions ($r = 0.68$; Table 5.2). The impact of salinity on P_n also differed substantially among barley cultivars, with salt-tolerant genotypes maintaining P_n 3-fold higher than the salt-sensitive ones.

Most cultivars had over 75% relative germination rate (Figure 5.1F) under saline conditions, with rather poor correlation between this trait and salt tolerance (Table 5.2). Plant height, survival rate, and tillering were also greatly limited by severe salinity (Figure 5.1C, E and H). Both plant height and survival rate correlated strongly ($P < 0.05$) with plant salt tolerance (estimated as grain yield; Table 5.2), but rather poorly with tillering.

5.3.2. Correlations between K^+ flux and physiological characteristics

In parallel experiments, net K^+ fluxes were measured from 3-d old seedlings after 1 h treatment with 80 mM NaCl. Fluxes were measured twice, during Trial 1 and Trial 2 (total sample size n = 8–13). The average flux values for those two trials are shown in Table 5.1, where genotypes are listed in descending order according to the magnitude of K^+ flux. Each genotype was also ranked based on its overall performance in the glasshouse experiments using six key physiological characteristics – relative grain yield, shoot biomass, height, P_n, survival rate and TSW – with an equal weight given to each of these parameters. From the Chapter 4, it was expected that the salt tolerance should correlate with a plant's ability to minimise NaCl-induced K^+ efflux from roots. Indeed, strong correlations were observed between the magnitude of net K^+ flux and relative grain yield (Figure 5.1A), shoot biomass (Figure 5.1B), plant height (Figure 5.1C), P_n (Figure 5.1D), survival rate (Figure 5.1E) and TSW (Figure 5.1G). Much poorer correlations were found between K^+ flux and either relative germination rate (Figure 5.1F) or tillering (Figure 5.1H). Exponential regression was used in Figure 5.1 as it fits better than linear regression in estimating the relationship between all the physiological parameters and K^+ efflux.

Table 5.1. Average grain yield and shoot biomass of control and salt-treated plants of barley varieties measured in consecutive trials (Trial 1 and Trial 2, respectively) over two seasons. The genotypes are listed in descending order according to the magnitude of net K^+ fluxes (mean values of 8 to 13 plants; efflux negative) and ranked based on their mean scores of six key physiological parameters: relative grain yield, shoot biomass, plant height, P_n, survival rate and TSW.

Cultivar name	*K^+ flux, nmol m^{-2} s^{-1}*	*Grain yield, g pot^{-1}*				*Biomass, g pot^{-1}*				
		Salinity		*Control*		*Salinity*		*Control*		
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	*Rank*
7204	-471±31	0	0	40±6	36±2	0.1±0.1	0.1±0.1	70±7	84±5	67
Yan 91048	-431±21	0.1±0.1	1.4±0.7	39±2	52±4	0.5±0.2	13.3±2.4	65±1	112±6	58
Franklin	-408±17	n.m.	0	n.m.	53±3	n.m.	0.8±0.1	n.m.	104±12	69
96-6404	-406±31	0.1±0.1	0	24 ±3	41±2	0.4±0.2	0.3±0.3	42±5	106±8	66
Yangsimai 3	-404±22	0	0	24±2	42±2	0.4±0.2	2.6±1.9	37±2	93±4	61
Sunong 133-2	-398±29	0.4±0.1	2.4±2.0	28±3	44±5	1.0±0.3	10.2±5.6	54±3	91±10	56
ZUG167	-395±23	0.1±0.0	0.4±0.3	42±2	56±5	0.5±0.1	7.1±2.6	67±2	113±8	61
Gairdner	-392±18	0	0	46±4	60±1	0.3±0.2	1.2±0.6	89±5	126±4	64
Lixi 143	-384±19	0.3±0.1	0.6±0.4	24±1	44±1	1.0±0.3	5.5±1.7	41±3	91±6	56
ZUG165	-380±34	1.2±0.2	1.5±1.5	43±2	53±6	2.7±0.2	5.5±3.1	64±5	109±10	54
YPSLDM	-378±39	n.a.	0	n.a.	43±4	1.2±1.1	0.3±0.1	56±3	108±4	64
ZUG31	-372±19	n.m.	0.1±0.1	n.m.	62±3	n.m.	1.7±1.2	n.m.	120±7	66

Yan 96219	-363±24	0	0	31±2	31±2	1.3±0.8	1.9±0.2	75±3	86±2	61
ZUG405	-354±19	2.0±0.2	1.4±0.8	41±3	45±1	4.0±1.1	8.4±3.0	60±4	99±7	48
ZUG403	-338±24	n.m.	0	n.m.	54±2	n.m.	0.6±0.2	n.m.	114±3	68
Hu 01-2946	-335±30	0.4±0.1	0.9±0.6	18±3	47±4	1.0±0.1	7.5±2.5	32±3	114±5	56
Sunong 0137	-334±25	3.1±0.2	2.1±2.0	43±2	37±4	3.9±0.2	19.1±8.8	78±3	89±7	50
Yan 95168	-325±10	0.8±0.1	0.1±0.1	44±4	38±3	1.6±0.1	1.9±0.6	60±6	92±4	59
Rugaozaoliuleng	-324±11	n.a.	0.8±0.5	n.a.	37±3	7.5±1.0	15.1±4.5	51±4	85±7	31
KA-4B	-319±10	0.7±0.2	3.4±1.3	33±3	44±2	1.7±0.6	12.6±3.5	59±5	89±7	50
Zheda 96-6	-314±18	0.8±0.2	1.0±0.8	11±1	35±3	2.5±1.1	9.5±2.7	27±4	99.5±7	53
Sunong22	-306±39	1.2±0.1	4.5±1.7	50±2	43±6	3.9±0.4	11.2±2.3	71±2	80.7±10	43
Yangnong 9001	-305±21	1.0±0.1	0.5±0.4	32±2	55±5	2.6±0.2	16.3±7.2	52±3	102±10	44
ZUG401	-303±24	1.2±0.2	0.7±0.4	44±3	50±3	2.8±0.2	5.1±1.1	69±3	108±6	48
Zhenongda 3	-300±10	0.4±0.2	2.4±1.4	40±1	35±3	1.6±0.4	8.6±3.4	58±2	73±5	46
ZUG25	-287±19	0.7±0.3	1.1±1.1	32±9	42±2	1.4±0.6	8.7±4.9	65±9	95±3	51
CXHJSLDM	-286±12	n.a.	10.1±6.8	n.a.	49±4	11.3±0.9	19.9±11.4	62±4	98±8	24
YWHKSLDM	-286±20	n.a.	8.4±4.0	n.a.	37±4	11.6±1.0	21.5±7.1	76±16	86±8	31
Yan 95221	-280±14	2.9±0.6	4.2±3.4	43±2	33±3	6.3±0.9	13.5±7.0	62±2	82±9	34
Aizao 3	-276±18	1.6±0.2	2.1±1.3	38±4	42±4	3.7±0.2	10.2±3.3	59±6	78±6	40
ZND 85-17	-273±20	0.8±0.2	1.3±0.8	20±2	38±1	1.8±0.4	9.8±3.1	36±3	86±5	46
ZUG707	-264±26	0.3±0.0	9.1±4.5	42±2	38±1	1.5±0.1	24.8±7.0	64±2	93±4	40

Yan 96116	-260±12	5.1±0.6	8.9±5.8	31±2	50±2	8.2±1.0	21.0±9.4	53±23	101±5	9
Yangyin 02	-258±12	n.a.	6.8±3.7	n.a.	44±2	8.8±0.5	16.8±7.8	66±2	95±5	15
Zhoumai 6	-252±12	3.7±0.6	16.8±7.0	47±1	48±2	8.7±0.6	38.1±10.3	74±2	98±4	6
Gobermadora	-250±14	7.2±0.4	2.4±1.1	38±5	36±1	13.4±1.2	8.6±2.3	80±4	81±5	20
ZUG9	-250±17	5.7±0.8	4.8±2.1	38±2	39±3	9.4±1.0	13.2±3.8	61±2	84±6	24
YYXTEL	-246±24	3.4±0.5	5.2±3.0	34±4	30±3	11.3±1.9	28.7±5.5	92±2	76±4	17
ZUG627	-243±8	1.3±0.2	2.7±1.1	36±7	45±4	3.0±0.3	19.5±4.5	57±8	99±10	36
Yan 91-269	-242±12	2.4±0.3	20.3±8.5	43±3	53±5	4.9±0.3	44.0±10.6	67±3	94±5	12
96 AC-13-11	-239±18	3.3±0.4	2.8±1.3	29±3	48±5	6.5±0.6	12.4±3.8	49±2	94±6	27
96AC14-16	-237±9	0.5±0.2	0.3±0.3	21±1	43±2	1.2±0.4	9.0±1.9	44±2	82±4	53
ZUG673	-228±17	5.9±1.1	3.3±3.3	34±3	46±3	11.5±0.5	12.6±6.6	56 ±5	92±8	20
Yangpi 1	-227±16	n.m.	1.7±2.0	n.m.	43±4	n.m.	8.2±2.6	n.m.	100±6	38
ZUG95	-227±15	3.6±0.5	11.9±1.0	39±2	50±3	6.1±0.4	26.3±2.1	68±2	99±10	20
AC Burman	-222±13	15.4±0.6	9.8±4.8	68±1	44±5	28.2±1.4	41.8±10.1	102±1	117±5	4
ZUG797	-218±14	3.2±0.1	11.9±5.0	37±1	43±2	6.2±0.1	32.2±6.5	54±2	84±5	5
Klages	-213±15	8.7±0.8	0.1±0.1	40±3	42±2	22.4±1.7	4.0±1.4	90±6	106±4	31
Aigan-4	-206±9	2.8±0.1	0.3±0.3	48±3	29±2	5.2±2.1	3.6±2.7	68±4	57±3	43
Su B9601	-204±21	3.3±0.3	13.3±6.9	37±5	39±1	6.7±0.3	32.3±11.7	58±56	84±7	12
KA-4A	-202±15	1.9±0.3	7.4±3.9	32±1	46±3	5.2±1.1	14.2±6.4	57±1	97±8	26
ZUG831	-201±13	4.2±0.8	12.3±4.6	42±2	50±4	7.7±0.8	32.5±5.8	71±1	108±8	12

YU6472	-198±9	n.m.	2.7±2.6	n.m.	52±2	n.m.	22.4±7.6	n.m.	99±6	20
Canada 110	-192±10	1.8±0.2	1.7±1.1	10±2	36±5	4.1±0.5	8.2±2.1	38±3	89±8	36
ZUG159	-189±11	n.a.	8.8±4.7	n.a.	48±1	8.3±0.5	36.0±6.6	51±45	114±3	26
Yan 89110	-186±10	0.7±0.1	10.8±4.0	31±3	42±2	2.3±0.3	35.5±9.7	57±2	95±4	31
ZUG161	-179±8	4.7±0.3	6.4±4.3	41±2	43±2	8.2±1.3	15.7±6.8	64±1	93±7	12
ZUG53	-178±13	n.a.	6.4±3.1	n.a.	37±8	7.7±0.4	27.4±2.9	45±4	94±12	20
Suyin 27	-175±11	3.2±0.3	7.9±3.1	25±3	40±5	7.5±0.7	24.6±7.2	43±4	91±10	8
ZUG673-2	-170±13	3.5±0.7	2.1±0.4	37±3	41±1	7.4±0.7	12.1±1.3	56±4	87±2	31
Yan 96001	-169±19	2.5±0.3	7.3±4.3	42±1	33±3	6.1±0.2	9.3±2.1	56±2	76±7	31
SUF0202	-164±10	4.5±0.3	5.7±3.2	43±2	39±3	8.1±0.3	18.5±11.0	64±2	97±7	16
Silengdamai	-161±15	n.a.	4.6±1.7	n.a.	41±4	5.2±0.4	20.0±6.5	73±3	91±8	37
Taixing 9425	-145±9	2.4±0.6	3.7±0.9	13±4	27±4	5.3±1.2	21.9±4.0	26±6	74±7	15
S252/Sunong 37	-143±13	1.8±0.2	5.3±2.5	27±2	39±3	4.5±0.6	14.0±4.3	40±3	84±67	40
Numar	-131±9	n.m.	21.5±6.5	n.m.	57±2	n.m.	41.0±7.6	n.m.	120±2	3
Hu 93-045	-116±10	5.3±0.8	7.9±1.5	32±6	49±2	8.4±1.0	29.8±7.0	48±5	109±3	8
CM72	-92±10	7.8±0.7	17.7±3.6	43±4	62±6	14.5±0.5	38.0±6.1	65±5	117±10	2
ZUG293	-89±6	n.m.	12.7±4.5	n.m.	36±4	n.m.	31.1±7.3	n.m.	98±6	1

Note: n.m. Grain yield and shoot biomass were *not measured* for those cultivars in Trial 1. n.a. Grain yield is *not available* for the winter cultivars in Trial 1.

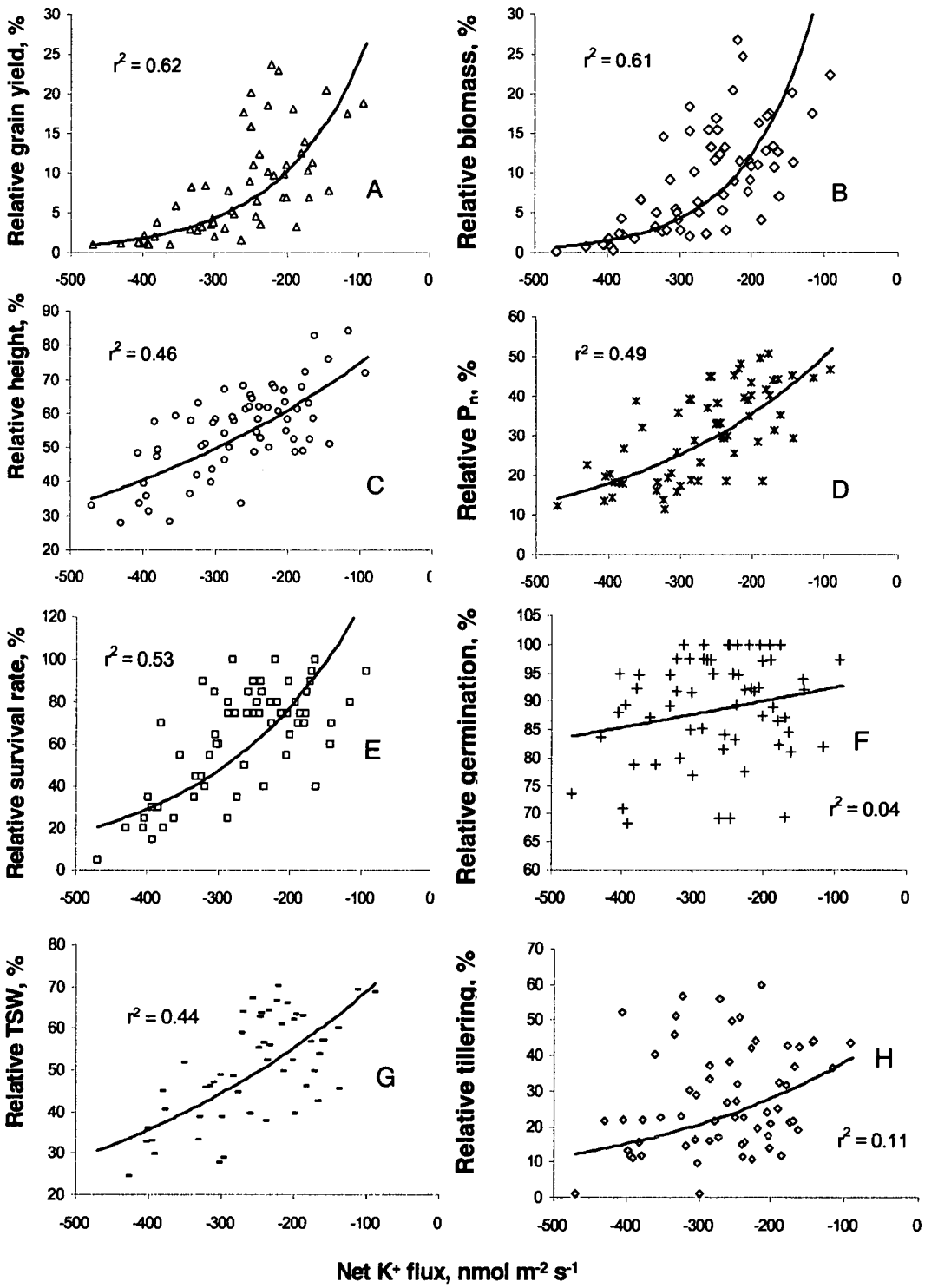

Figure 5.1. Correlation between the relative (% of control) grain yield (A), shoot biomass (B), height (C), CO_2 assimilation (D), survival rate (E), germination rate (F), thousand seeds weight (G), and tillering (H) measured in glasshouse experiments and net K^+ flux measured from the mature zone of 3-d old roots after 1 h of 80 mM NaCl treatment. Points are pooled from physiological parameters in Trial 1 and average K^+ flux of 8 to 13 seedlings in both trials.

Table 5.2. Linear correlation (r) between various physiological characteristics (% of the control) measured from 62 barley genotypes in glasshouse during Trial 1 and laboratory experiments (K^+ flux).

Parameter	*Grain yield*	*K^+ flux*	*Biomass*	*Survival*	*Height*	*P_n*	*TSW*	*Tillering*
K^+ flux	0.67**							
Biomass	0.96**	0.69**						
Survival	0.65**	0.70**	0.74**					
Height	0.70**	0.69**	0.61**	0.51**				
P_n	0.68**	0.69**	0.65**	0.48**	0.50**			
TSW	0.72**	0.70**	0.70**	0.63**	0.74**	0.48**		
Tillering	0.48**	0.26*	0.51**	0.16	0.23	0.25*	0.33*	
Germination	0.29*	0.21	0.31*	0.33**	0.16	0.02	0.38**	0.2

Note: Significant at * $P < 0.05$ and ** $P < 0.01$ level

Further evidence of the strong correlations between the magnitude of K^+ flux and the plant growth and yield responses is shown in Figure 5.2. All the genotypes were grouped according to their K^+ flux values into 50 nmol m^{-2} s^{-1} intervals, and an average shoot biomass and grain yield for each group were plotted against K^+ flux for each season (Figure 5.2). Cultivars with K^+ flux above -300 nmol m^{-2} s^{-1} gained significantly more ($P < 0.05$) shoot biomass and grain yield than those below -300 nmol m^{-2} s^{-1} (Figure 5.2A, and C). No dramatic differences were observed among the control groups, although the most salt-tolerant varieties had a tendency to have slightly smaller yield in Trial 1 than other groups (Figure 5.2B, and D). Overall, it appeared that cultivars with increased shoot biomass (Figure 5.2A) and grain yield (Figure 5.2C) had an enhanced ability to prevent NaCl-induced K^+ efflux from roots, suggesting that a smaller K^+ efflux is related to a higher salt tolerance.

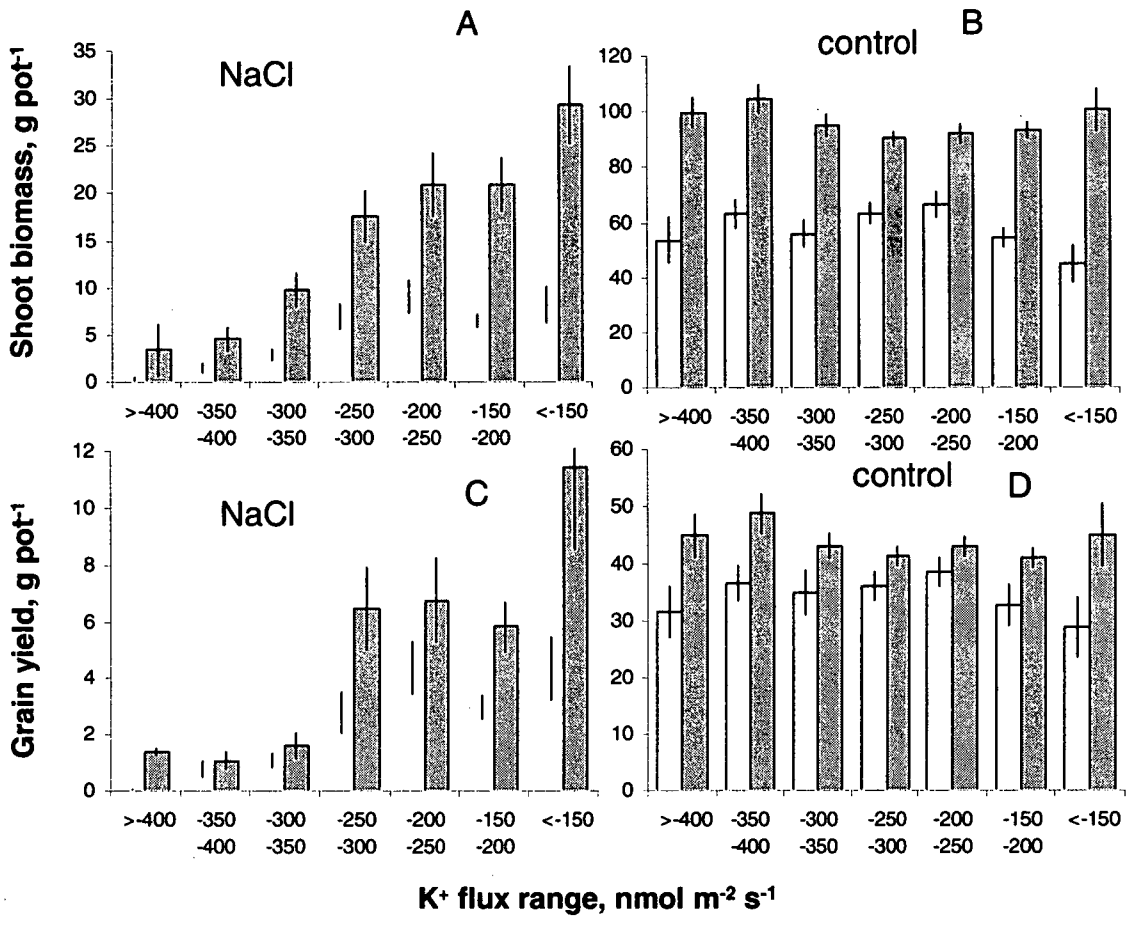

Figure 5.2. Average shoot biomass and grain yield of cultivars, grouped according to their net K^+ flux values into 50 nmol m^{-2} s^{-1} intervals, and plotted against K^+ flux for each season (Trial 1, open bars; Trial 2, closed bars) in saline (A and C) and control (B and D) conditions. Within each season, different *lowercase* letters indicate significant difference at $P < 0.05$. Means ± SE (n = 4).

5.3.3. Leaf Na^+ and K^+ analysis

Despite the generally strong correlation, a substantial variability in plant grain yield and shoot biomass was apparent, especially in the middle range (-200 to -350 nmol m^{-2} s^{-1}) of K^+ fluxes (Figure 5.3A), with a much larger number of deviations than from those at both ends (the standard deviations for each of three regions, shown in Figure 5.3A, were 1.44, 7.21 and 3.96 g pot^{-1}, respectively). As a result, a several-fold difference in growth and yield could be observed between genotypes showing similar K^+ flux in this range (Figure 5.1A and 5.3A).

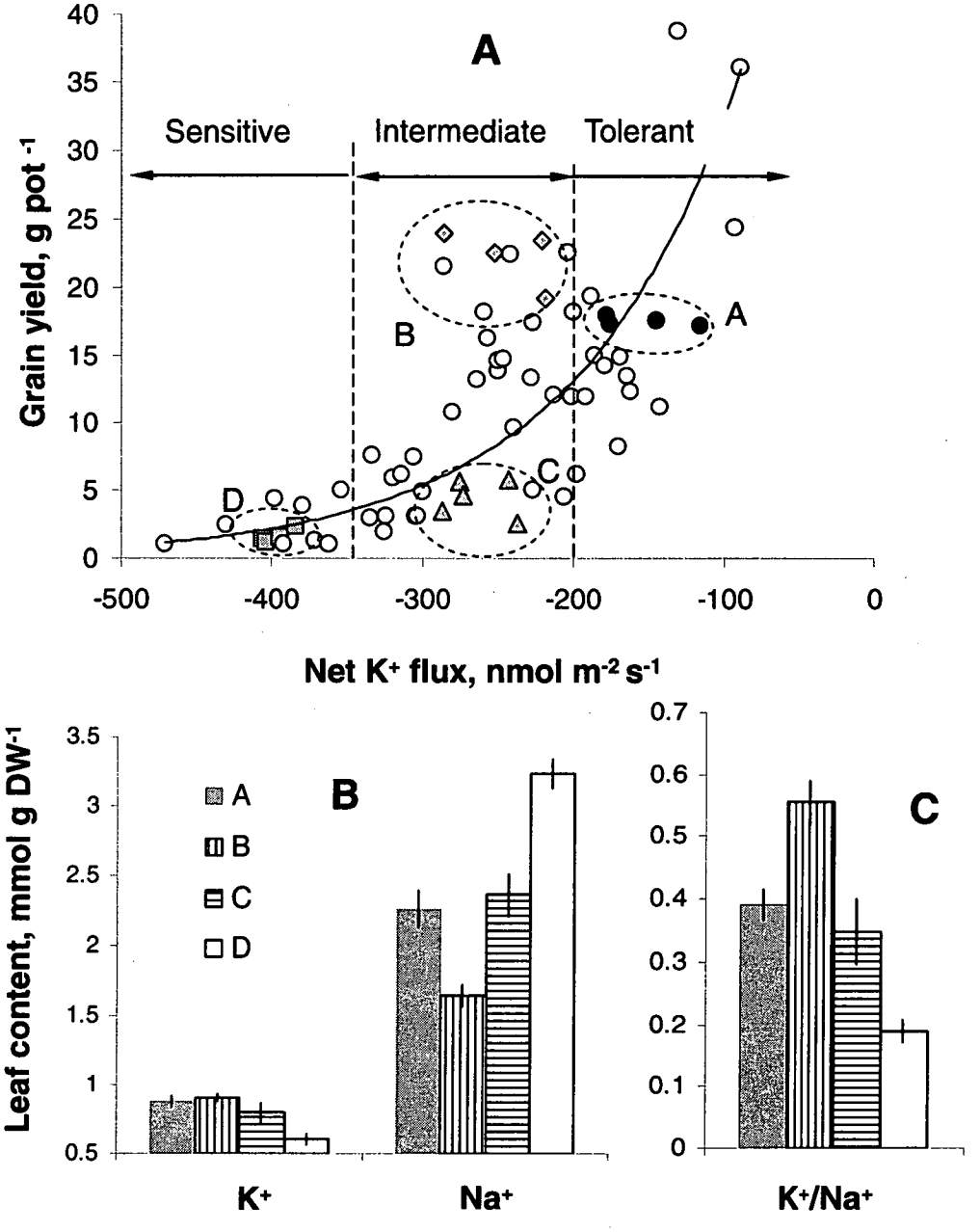

Figure 5.3. (A) – correlation between barley grain yield and NaCl-induced net K^+ flux measured from 3-d old seedlings. Dashed circles indicate four groups (labelled A to D) selected for further nutrient analysis. Group A (black discs) – high yielding plants with good K^+ retaining ability; group B (grey diamonds) – high yielding plants from the "middle-range" K^+ retaining ability; group C (grey triangles) – low yielding plants from the "middle-range" K^+ retaining ability; group D (grey squares) – low yielding plants with poor K^+ retaining ability. (B) – average leaf K^+ and Na^+ concentration and (C) – K^+/Na^+ ratio in plants from the groups A to D. Different *lowercase* letters in panels B and C indicate significant difference at $P < 0.01$. Means ± SE (n = 4).

To shed some light on the nature of this 'middle range' variability, an analysis of Na^+ and K^+ concentration was performed on four groups of selected genotypes (Figure 5.3A; Table 5.3). Cultivars chosen are those which have been included in both trials and which showed consistency between Trial 1 and Trial 2. These are labelled with the shaded symbols in Figure 5.3A. Average grain yields over the two seasons were plotted against net K^+ flux (Figure 5.3A; includes also the seven cultivars grown only in Trial 2). Of the four selected groups, salt-tolerant (group A) and -sensitive (group D) varieties, clearly showed the inverse relationship between K^+ efflux and salt tolerance. Leaf nutrient analysis showed that in group D, leaf K^+ concentration was significantly lower than that of groups A and B; while group D also showed the highest average leaf Na^+ concentration, almost double that for group A (Figure 5.3B). For the two groups selected from the middle range, group B plants performed much better than average, and group C plants were below the average trendline. The major difference between two groups (B and C) was in the Na^+ concentration and K^+/Na^+ ratio. Despite showing the same (moderate) K^+ efflux upon NaCl treatment, cultivars of group B had the highest K^+/Na^+ ratio, directly reflected by the highest grain yield (Figure 5.3A, and C) as well as other parameters (Figure 5.1) among the four groups. Groups A and C had similar K^+/Na^+ ratios, but were strikingly different in grain yield, probably as a result of their different ability to retain K^+ (judged by K^+ flux measurements; Table 5.1). The difference in leaf K^+ between group B and C plants was not significant, which is consistent with their similar rate of K^+ efflux from the root (Table 5.1; Figure 5.3A). Group D plants were not capable of either restricting Na^+ accumulation in leaf tissues or preventing K^+ loss from the roots, which led to the lowest yields in this study. Strong correlations ($r^2 > 0.5$, $P < 0.001$) were also found between Na^+/K^+ ratio and net K^+ flux, and between Na^+/K^+ ratio and plant shoot biomass (Figure 5.4A and B) for all plants.

Table 5.3. Na^+ and K^+ concentration in leaves of salt treated plants from four groups as denoted in Figure 5.3A. Means ± SE (n = 4 replicates per cultivar). Ranking was based on six physiological parameters in both Trial 1 and Trial 2 (see text for details).

Cultivar name	*Group*	*K^+, mmol g DW^{-1}*	*Na^+, mmol g DW^{-1}*	*K^+/Na^+ ratio*	*Rank*
Suyin27	A	0.85±0.004	2.45±0.014	0.35±0.007	8
Taixing9425	A	0.79±0.003	1.90±0.028	0.42±0.010	15
Hu93-045	A	0.89±0.010	2.49±0.016	0.36±0.005	8
ZUG53	A	1.00±0.006	2.22±0.017	0.45±0.003	20
YWHKSLDM	B	0.86±0.014	1.53±0.014	0.56±0.027	31
AC Burman	B	0.96±0.016	1.50±0.027	0.64±0.007	4
Zhoumai6	B	0.86±0.004	1.85±0.024	0.46±0.016	6
ZUG797	B	0.95±0.006	1.70±0.014	0.56±0.013	5
ZND85-17	C	0.83±0.002	2.38±0.029	0.35±0.008	46
Aizao3	C	0.98±0.003	2.08±0.027	0.47±0.011	40
96AC14-16	C	0.74±0.010	2.69±0.022	0.28±0.003	53
ZUG25	C	0.85±0.005	2.13±0.028	0.40±0.014	51
ZUG627	C	0.63±0.011	2.55±0.033	0.25±0.002	36
Yangsimai3	D	0.56±0.014	3.15±0.086	0.18±0.002	61
96-6404	D	0.52±0.002	3.54±0.045	0.15±0.002	65
Lixi143	D	0.61±0.005	3.14±0.064	0.19±0.008	56
ZUG167	D	0.73±0.014	3.09±0.025	0.24±0.008	61

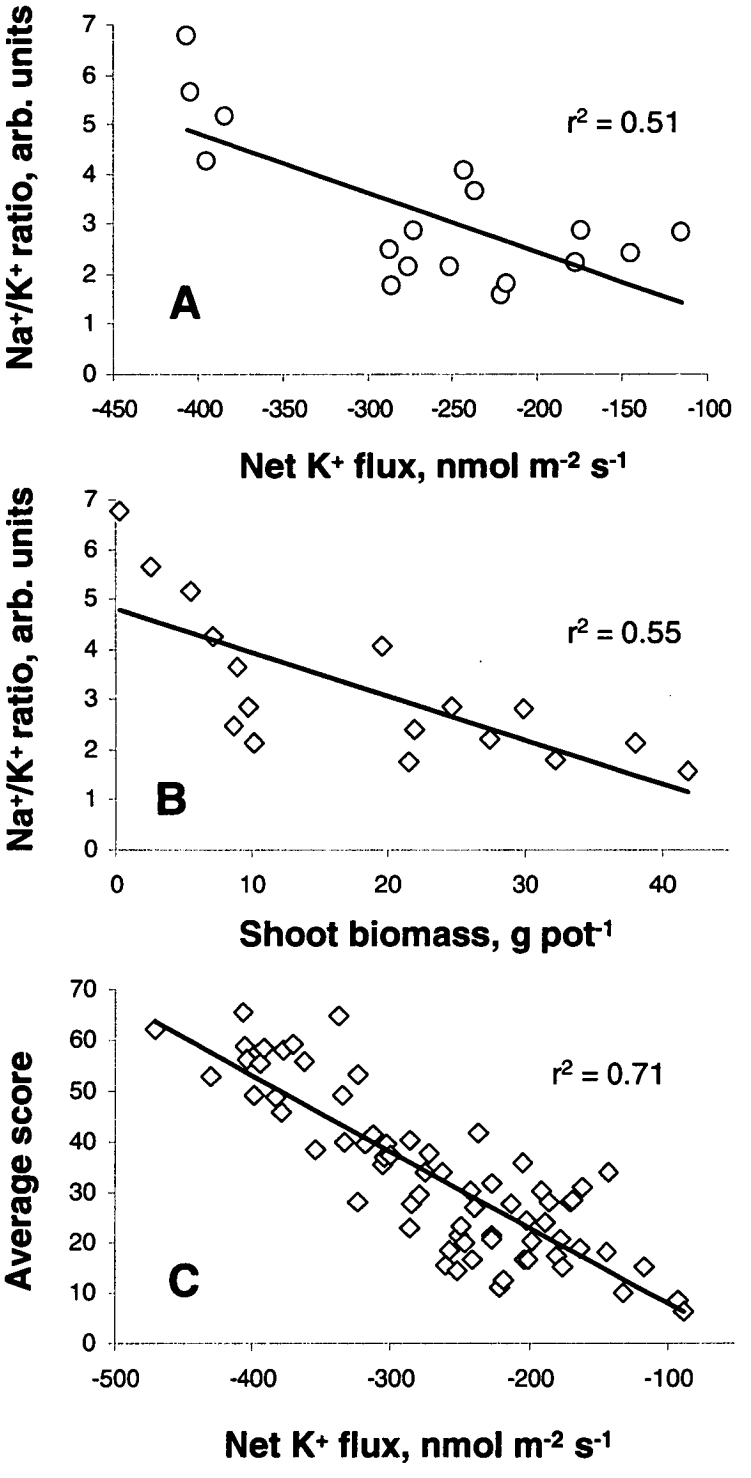

Figure 5.4. Linear regression of Na^+/K^+ ratio on net K^+ flux (A) and shoot biomass (B). Data were pooled from Na^+/K^+ ratio of 17 cultivars (listed in Table 5.2) and their corresponding net K^+ flux and shoot biomass. (C) – a linear regression of the average ranking score (based on six physiological characteristics – relative grain yield, shoot biomass, height, P_n, survival rate and TSW over the two trials; with 1 being the highest and 69 the lowest score) of salt tolerance on net K^+ flux. All the correlations are highly significant at $P < 0.001$.

5.4. Discussion

5.4.1. Genetic diversity of salt tolerance in barley

A wide range of cultivars was used in this study; these included winter *vs* spring, feed *vs* malt, six-rowed *vs* two-rowed, husked *vs* huskless, awned *vs* awnless barley varieties. It is not surprising, therefore, that plant physiological responses to NaCl treatment varied dramatically between genotypes. Varietal differences for yield in saline conditions have been previously reported in several studies on cereals under both glasshouse and field conditions (Greenway 1962; Richards et al. 1987; Slavich et al. 1990; Royo and Aragüés 1999). In this study, plant responses among contrasting genotypes varied from no grain yield (e.g. 7204 and Yan 96219) in salt-sensitive cultivars to ~80% yield reduction in salt-tolerant cultivars (such as CM72 and Numar; Table 5.1) under severe saline conditions (30 dS m^{-1}). Such genetic diversity of responses opens good prospects for barley breeding for salt tolerance.

5.4.2. Assessment of salt tolerance using various physiological traits

As salinity affects almost every aspect of the physiology and biochemistry of the plant, it was important to look at the suitability of using various physiological traits as indicators of salt tolerance.

Germination rate is by far the easiest character to measure, but is the least likely to predict the ability of plants to grow in saline soil (Table 5.2). My results are consistent with other reports in the literature. No correlation was observed between salt tolerance at germination and that at the seedling or later growth stages in tomato (Foolad and Lin 1997) and barley (Mano and Takeda 1997a). Equally, no significant correlation was found between seed germination and grain yield in wheat (Ashraf and McNeilly 1988). Salt tolerance appears to vary with growth stage (Storey and Wyn Jones 1978; Heenan et al. 1988). Barley is most sensitive to salinity at the seedling stage, and exhibits increased tolerance with age (Greenway 1965). This can explain why, in my experiments, relatively salt-tolerant genotypes gained a higher grain yield but most salt-sensitive ones only grew up to the seedling stage.

Grain yield is the ultimate criterion of salt tolerance. The major hurdle is the

duration of experiments (4–6 months; replicated over two seasons) and the large variability of growth conditions in the field. This makes screening a large number of genotypes for salt tolerance in the field an extremely difficult, if not impossible, task. Thus, field experiments may be more appropriate in the final stages of a breeding programme, rather than at initial stages when screening and selection for elite germplasm or for specific traits are best done under controlled conditions.

Survival rate, as a selection criterion for salt tolerance (Sayed 1985) is a rapid and relatively simple method. While other authors found only a weak relationship between survival rate in salinised nutrient solution and yield in saline soils (Richards et al. 1987), results reported here have suggested that this parameter was correlated strongly with both the magnitude of K^+ efflux (as a measure of salt tolerance) and grain yield (Table 5.2).

CO_2 assimilation reduction strongly correlated with plant grain yield (Table 5.2) and hence salt tolerance. Munns et al. (2006) concluded that better photosynthetic capacity (and the consequent greater salt tolerance) was due to high K^+, low Na^+, and the resulting high K^+/Na^+ ratio in the cytoplasm of mesophyll cells for both durum wheat and barley. However, the feasibility of CO_2 measurements as a screening tool is somewhat questionable, as it took almost a week to measure all 70 cultivars in glasshouse experiments. In addition, it took two months of treatment and subsequent plant growth in the glasshouse prior to that. As a possible alternative, measurements of stomatal conductance by either viscous flow porometry or indirectly by leaf temperature (thermal imaging) can be a promising way of measuring several hundred genotypes per day (James et al. 2002).

5.4.3. Combination of physiological parameters

It was previously suggested (Shannon and Noble 1990; Flowers and Yeo 1995; Colmer et al. 2005) that screening for salt tolerance should use physiological traits in combination, rather than simply based on yield or yield components. In my experiments, six physiological traits have been employed (relative grain yield, shoot biomass, plant height, P_n, survival rate and TSW). Despite some variation in the overall ranking for each of these parameters, their combination (averaged ranking score based on the equal weighting for each of these) appears to be a rather reliable

way to evaluate salt tolerance in barley (Table 5.1). As a result, the correlation (r^2 = 0.71, $P < 0.001$; Figure 5.4C) between the overall ranking score and K^+ flux was much higher than correlation between any individual parameter and K^+ flux.

5.4.4. NaCl-induced K^+ efflux as a measure of salt tolerance in barley

Potassium efflux measured from the mature zone (10 to 20 mm from the root tip) of the root epidermis of 3-d old barley seedlings after 1 h of NaCl treatment showed a strong correlation (r^2 between 0.44 and 0.62) with the relative grain yield, shoot weight, plant height, rate of P_n, survival rate and TSW measured in glasshouse experiments after 4 to 6 months of saline treatment. Most of those parameters are conventionally employed as indicators of plant salt tolerance (Greenway 1962; Sayed 1985; Asch et al. 2000; James et al. 2002). For the nearly 70 genotypes, and such a polygenic trait as salt tolerance, this is a remarkably good correlation. A plethora of different mechanisms encodes salt tolerance including Na^+ exclusion by roots (Liu and Zhu 1997; Zhu et al. 1998), Na^+ compartmentation in vacuoles (Zhang and Blumwald 2001; Zhang et al. 2001), K^+/Na^+ discrimination during xylem loading (Munns 2000; Lindsay et al. 2004), increased levels of compatible solutes (Hasegawa et al. 2000) etc. In the light of all the above, it would hardly be expected that barley genotypes bred for over ten thousand years (Badr et al. 2000) will possess only one mechanism to cope with salinity. Nonetheless, an r^2 value above 0.5 between each growth/yield parameter and K^+ flux suggests that efficient retention of K^+ in plant tissues is the dominant strategy responsible for over 50% of the variation in salt tolerance in barley.

5.4.5. MIFE K^+ efflux measurements as a screening tool

Net K^+ fluxes were measured under laboratory conditions from more than 730 individual seedlings using the MIFE technique, following the screening routine developed in previous work (Chapter 4, Chen et al. 2005). On average, with measuring and preparing time at about 8 min per sample, up to 40 plants per day were screened by the MIFE technique, so all 70 genotypes were done in about 4 weeks.

Despite being skill-demanding, this is nonetheless much quicker than growing plants for 4 to 6 months and less time-consuming than the labourious procedure of harvesting and evaluating grain yield. The MIFE technique can distinguish contrasting barley varieties differing substantially in salt tolerance. If those varieties deviating from the exponential trendline (~ 15% of total number; mostly in the moderate range of fluxes, between -200 and -350 nmol $m^{-2} s^{-1}$) are excluded from analysis, correlation values are much higher ($r^2 > 0.6$; data not shown). This indicates that the MIFE technique alone can be used to distinguish about 85% of barley cultivars for their salt tolerance in a breeding programme.

It should be also kept in mind that for such a polygenic trait as salt tolerance (Flowers 2004), it would be far too naive to assume that any single method, regardless of how sensitive it is, will have a 100% predictive value. Accordingly, if the screening was based on one particular criterion only, there will always be a danger of "missing" some salt-tolerant cultivars. In this case, some barley cultivars did not show low K^+ efflux, yet showed some considerable residual yield/growth in response to salt (those from group B; Figure 5.3A). This appears to be related to the ability of those cultivars to restrict entry of Na into the shoot. These findings emphasised the need for a multilateral approach and use of an array of screening methods to deal with physiological complexity of salt tolerance mechanisms. This issue is further discussed in the following section.

5.4.6. Tissue K^+/Na^+ ratio as a critical feature for salt tolerance in barley

Tissue K^+/Na^+ ratio has often been named as central to salt tolerance in various plant species (Chhipa and Lal 1995; Dubcovsky et al. 1996; Maathuis and Amtmann 1999; Asch et al. 2000). Consequently, K^+/Na^+ discrimination has been subject to QTL analysis for salt tolerance in some cereals (Koyama et al. 2001; Cattivelli et al. 2002; Lin et al. 2004; Lindsay et al. 2004). The use of QTLs in a plant breeding programme depends not only on the importance of the traits but also on the use of those showing high heritability in the population. Previous reports have suggested that the overall K^+/Na^+ ratio is heritable, at least in some species (García et al. 1997).

In this study, the largest variability was in the 'middle K^+ flux range' with some

genotypes having 3-fold higher yield showing apparently the same K^+ efflux (Figure 5.3A). This variation was attributed to better ability of plants from group B to prevent Na^+ accumulation in the shoot (Figure 5.3B). As a result, these plants had the highest K^+/Na^+ ratio in leaf tissues, even higher than plants from group A (Figure 5.3C). Due to the complexity of the whole plant nutrient uptake process, it is not clear where such exclusion takes place. The main possibilities are: (1) exclusion of Na^+ from uptake by roots; (2) prevention of Na^+ loading into the xylem; and (3) control of xylem unloading in leaves. Answering this question is outside of the scope of this chapter and remains an issue for future studies. Regardless of the mechanisms, plants with better ability to exclude Na^+ from leaves had the highest K^+/Na^+ ratio. For example, plants in group B had even higher K^+/Na^+ than group A, despite having a larger K^+ leakage. This emphasises again the importance of maintaining an optimal K^+/Na^+ ratio as a key determinant of plant salt tolerance (Figure 5.4B), at least in barley. Thus, it appears that the "ideal" barley genotype should not only possess an ability to retain K^+ efficiently in plant roots under saline conditions, but also have a means of preventing Na^+ accumulation in the shoot. Thus, further breeding efforts should be concentrated on identifying, characterising and localising the genes encoding these two traits in the barley genome.

In practical terms, selection of plants with higher K^+/Na^+ ratio in their tissues may be sufficient to pick up salt-tolerant genotypes. However, glasshouse screening of a large (hundreds of accessions) number of genotypes based on tissue K^+ and Na^+ analysis is extremely time-, labour- and money-costly exercise. MIFE measurements on young seedlings may be an ideal solution, assuming both K^+ and Na^+ fluxes are measured concurrently. The major limiting factor here is that all available Na^+ ionophores have rather poor selectivity (Carden et al. 2001; Chen et al. 2005; Chen et al. unpublished data) and thus are not suitable for this purpose. Once this problem is solved, a highly accurate and convenient screening method should become available to breeders.

Chapter 6. Inheritance of K^+ Efflux Trait and Salt Tolerance in Barley

6.1. Introduction

Genetic studies have been conducted using various criteria. High heritability of salt tolerance was reported based on Na^+ and K^+ uptake and K^+/Na^+ ratio in rice (Gregorio and Senadhira 1993), seed germination in barley (Mano and Takeda 1997a, b), shoot growth in lucerne (Noble et al. 1984), shoot and root growth in pearl millet (Ashraf and McNeilly 1992), and root length of seedlings in several grass species (Ashraf et al. 1986). Mano and Takeda (1997a, b) found that salt tolerance of barley at the germination stage was controlled by over-dominant alleles and non-additive genetic variance was larger than additive genetic variance, while at the seedling stage, tolerance was predominantly controlled by additive genes, with also some effects of dominance. Similar results in rice were reported by Gregorio and Senadhira (1993), based on Na^+ and K^+ uptake and K^+/Na^+ ratio, where salt tolerance was controlled by both additive and dominant gene effects. Moeljopawiro and Ikehashi (1981) reported that, based on divergent selection, the tolerance was a quantitative trait exhibiting additive, dominance and overdominance gene effects and was under polygenic control.

Barley has a relatively high level of tolerance to salinity than other cereal crops (Lessani and Marschner 1978; Shannon 1997; Munns et al. 2006). Varietal differences in response of barley to high salinity have been reported by Donovan and Day (1969). To bring salinity tolerance into commercial barley varieties, it is necessary to find genes for salinity tolerance in barley germplasm, to find a reliable screening method and to investigate the genetic behaviour of salt tolerance. In Chapter 4, a strong correlation was shown between the NaCl-induced root K^+ flux, measured by the MIFE technique, and salt tolerance using seven barley cultivars contrasting in salinity tolerance (Chen et al. 2005). The experiment was extended to 70 barley cultivars and the net K^+ flux from roots of 3-d old seedling of each cultivar, after salinity treatment, was significantly correlated ($P < 0.001$) with relative grain yield, shoot biomass, plant height, P_n, survival rate, and seed weight (Chapter 5, Chen et al. 2007). In this work, the heritability of the above K^+ flux trait as an indicator of salt tolerance was studied.

Six varieties with different salinity tolerance were selected to make crosses in a half diallel pattern to study the genetic behaviour of salt tolerance in barley. The results suggest a high heritability for salt tolerance in barley and show that plants could be effectively selected and bred for salt tolerance based on the NaCl-induced root K^+ flux measurements.

6.2. Materials and methods

6.2.1. Plant materials

Six barley cultivars, CM72 and Numar (salt-tolerant), YU6472 and Yan90260 (medium salt-tolerant) and Gairdner and Franklin (salt-sensitive) were used to make half of the possible crosses (without the reciprocal crosses) between them. Glasshouse experiments were carried out at two locations, Hobart (the second large-scale glasshouse trial in Chapter 5) and Launceston in Tasmania, for yield comparison for six parents, in 2004/05 summer and 2005/06 summer, respectively. Each cultivar was grown in pots filled with either potting mixture or potting mixture with salt (equivalent to 30 dS m^{-1}). Five plants were grown in each pot and the yield per pot was measured after harvesting.

6.2.2. Experimental protocol for K^+ flux measurements

K^+ fluxes were measured five to ten seedlings of six barley cultivars and F_1s, 40 to 120 seeds of F_2s, BC_1s, and BC_2s. Details of K^+ flux measurements are in 3.3.2.

6.3. Results

6.3.1. Salinity tolerance of selected parents

Glasshouse experiments have confirmed the previous observations that CM72 and Numar were tolerant to salt while Franklin and Gairdner were salt-susceptible. Both YU6472 and Yan90260 were introduced from China and showed medium tolerance to salinity. Compared to the controls, the percentages of yield loss under saline

conditions were 67 to 69% for two salt-tolerant varieties, 81 to 82% for medium salt-tolerant varieties and 96 to 98% for salt-susceptible varieties (Figure 6.1). The difference in salinity tolerance for different cultivars in the early growth stage can be clearly seen in Figure 6.2, in which the salt-tolerant cultivar CM72 was much healthier than the salt-susceptible cultivar Franklin. The six parents also differed in K^+ flux measured from root mature region of 3-d old barley roots after 1 h pre-treatment in 80 mM NaCl (Table 6.1, Figure 6.3) and the root K^+ flux measurements were closely related to the relative yield under saline conditions (Figure 6.1).

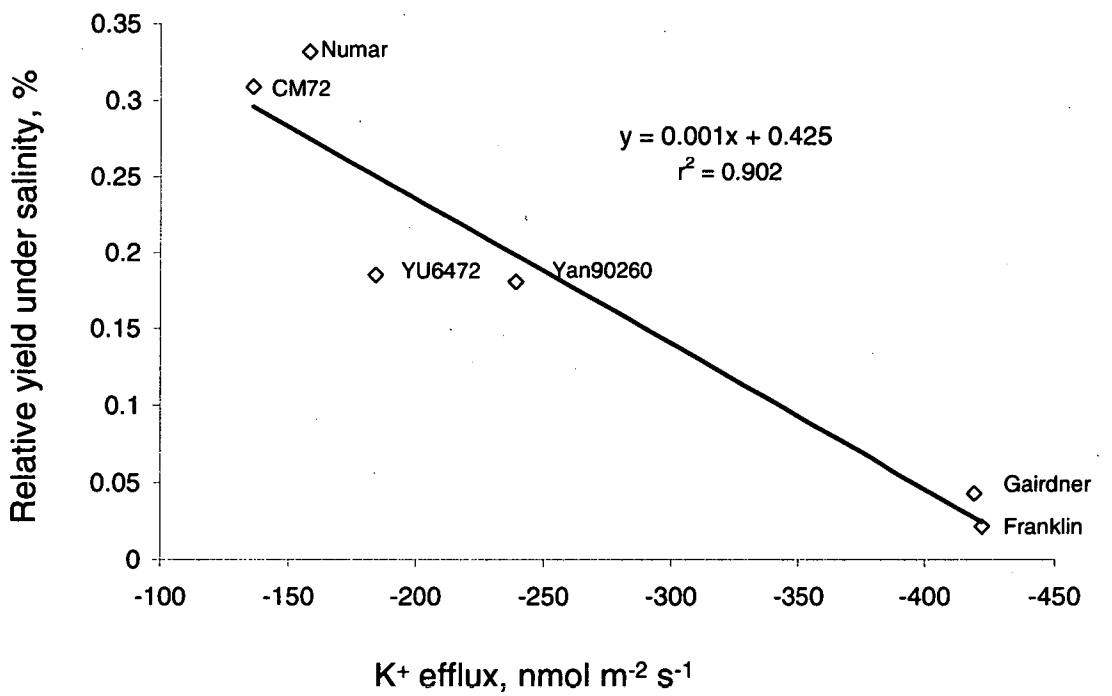

Figure 6.1. Correlations between NaCl-induced K^+ flux measured from mature region of 3-d old barley roots after 1 h pretreatment in 80 mM NaCl and the relative yield (average yield from experiments in Hobart and Launceston, Tasmania) under salt stress of six parents.

6.3.2. Root K^+ flux after salt treatment

Table 6.1 shows the mean root K^+ flux after salt treatment of six parents, their 15 F_1 progenies and 15 F_2 populations. Significant differences were found between different parents or F_1 populations (Table 6.2). The significant correlations between the root K^+ flux values of both F_1s and F_2s and the midparent values are shown in Figure 6.4. The average values of root K^+ flux of F_2 populations were significantly higher (i.e. less efflux) than the mid-parent values, indicating the dominance of salt tolerance (Figure 6.4). However, the average values of root K^+ flux of F_1 progenies were very close to the mid-parent values and did not show any dominance due to the weak development of F_1 seeds, which will be discussed later. The K^+ fluxes of all the F_2 populations showed near-normal distribution but with a slight skew towards the salt-tolerant variety. The distribution of an F_2 population from a typical cross between salt-tolerant (CM72) and -sensitive (Gairdner) cultivars skewed slightly towards the salt-tolerant cultivar (Figure 6.5).

Two crosses between the salt-tolerant cultivar CM72 and salt-susceptible cultivars Gairdner or Franklin were backcrossed to their parents. The distributions of the K^+ fluxes of the F_2, BC_1 and BC_2 populations from the cross between CM72 and Franklin are shown in Figure 6.6. Again, all the populations showed skew towards the salt-tolerant variety, indicating the existence of dominance of the salt tolerance genes.

Table 6.1. Half diallel data of K^+ flux measured from mature region of 3-d old barley roots after 1 h pre-treatment in 80 mM NaCl. Vr and Wr of each array in the diallel table were also calculated.

		CM72	*Numar*	*YU6472*	*Yan90260*	*Gairdner*	*Franklin*	*Mean*	*Vr*	*Wr*
CM72	F_1	-108	-134	-247	-216	-269	-186	-193	3995	5407
	F_2		-55	-157	-101	-224	-199	-141	4120	6839
Numar	F_1		-146	-204	-161	-279	-232	-193	3158	6410
	F_2			-199	-150	-220	-193	-161	3491	5747
YU6472	F_1			-215	-231	-303	-249	-242	1227	3012
	F_2				-195	-242	-226	-206	861	3134
Yan90260	F_1				-230	-335	-298	-245	3828	6885
	F_2					-247	-258	-197	3773	6709
Gairdner	F_1					-409	-388	-331	3331	6903
	F_2						-329	-279	5669	8544
Franklin	F_1						-374	-288	6508	9597
	F_2							-263	5384	8368
	F_1							Mean	3675	6369
	F_2								3883	6557

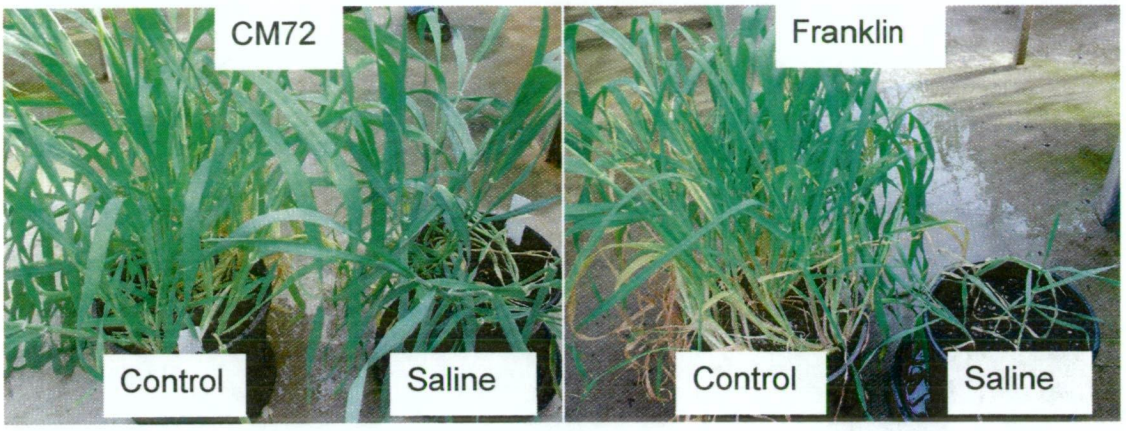

Figure 6.2. Effect of salinity on plant early growth of CM72 (left) Franklin (right). For each variety, plants in the right pot are salt-treated.

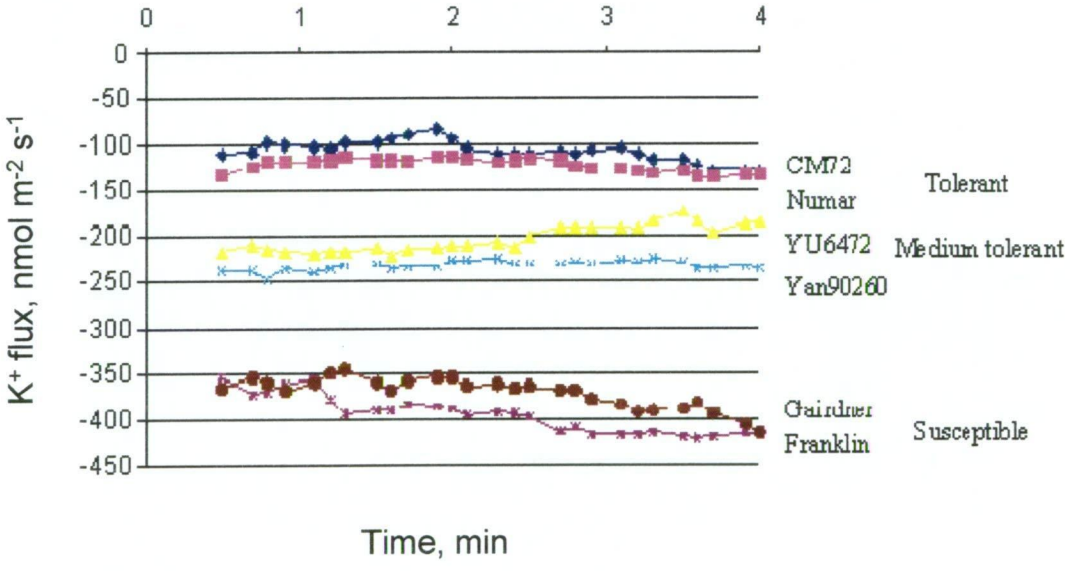

Figure 6.3. Steady-state K^+ fluxes of six parents after 1 h pre-treatment in 80 mM NaCl. Each point is the average of 10 measurements. For all MIFE measurements, the sign convention is "efflux negative".

Table 6.2. ANOVA of Parents and F_1 (for data shown in Table 6.1)

Source of Variation	*SS*	*df*	*MS*	*F*
Variety/cross	1892485	20	94624	30**
Error	641215	202	3174	
Total	2533700	222		

** Significant at $P < 0.01$ level.

6.3.3. Model test of the progenies

Root K^+ efflux of all the parents, F_1 and F_2 populations in response to NaCl treatment is shown in Table 6.1. Vr and Wr of each array in the diallel table were calculated and Wr was plotted against Vr (Figure 6.7). The slopes of the regression lines of both F_1 and F_2 generations were slightly higher than 1 even though only that of the F_2 generation differed significantly from 1, indicating the existence of non-additive dominance effects. The positions of the array points suggest the distribution of dominant and recessive genes in the parental arrays. The lowest point is from the YU6472 array, indicating that YU6472 had the largest number of dominant alleles (medium salt tolerance genes), while the highest is from the Franklin array which carried the largest number of recessive alleles (salt-sensitive genes).

According to the F_1 generation (Table 6.1), the genetically additive component of variation (D) was 13642 and the dominance component of variation (H) was 1056. The D and H calculated from F_2 generation were 13796 and 3470, respectively, which are similar to that from F_1 except for higher H. The H calculated from F_2 generation was multiplied by 2 since the dominance effect in F_2 is reduced by a half. Even though the estimation of both D and H may be biased due to the possible existence of non-additive-dominance effects, the additive effect still comprised the major part of genetic variation, which can also be seen from the distribution of F_2 populations and the correlations between midparent values and F_1 and F_2 progenies.

The genetic model was also examined with the *A*, *B*, and *C* scaling test and joint scaling test, using root K^+ flux of six generations (P_1, P_2, F_1, F_2, BC_1, and BC_2) from crosses of CM72 × Gairdner and CM72 × Franklin (Table 3). The *A*, *B*, and *C* scaling test showed that at least one of the *A*, *B*, and *C* values from both crosses differed significantly from 0, indicating the inadequacy of the additive-dominance model (Mather and Jinks 1977). A further joint scaling test indicated the inadequacy of the additive-dominance model with the χ^2 of both crosses at $P < 0.01$.

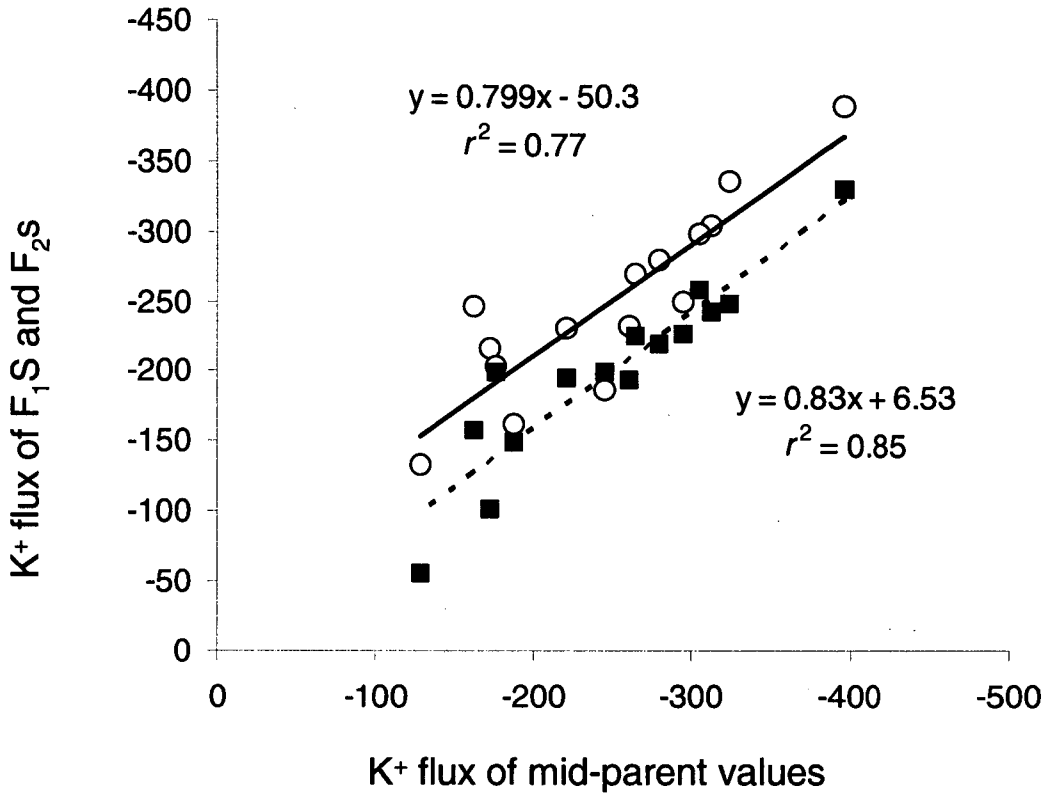

Figure 6.4. Correlations between the NaCl-induced K^+ flux of mid-parent value and their offsprings (F_1: hollow round and solid line; F_2: solid square and dashed line)

Table 6.3. Observed and expected mean of various generations and joint scaling test of CM72/Gairdner and CM72/Franklin with regard to K^+ flux measured from mature region of 3-d barley roots after 1 h pretreatment in 80 mM NaCl.

	Parameter	*CM72/Gairdner*	*CM72/Franklin*
A, *B*, and *C* scaling test	*A*	-46±44	-120±42
	B	104±52	19±41
	C	197±80	79±56
Joint scaling test	Joint (χ^2)	24	16
	P	<0.001	0.01–0.001

6.3.4. Heritability of salt tolerance based on the root K^+ flux after salt treatment

The heritability of salt tolerance was estimated using different methods and generations. In general, very high heritability was found for this trait, based on the root K^+ flux after salt treatment (Table 6.4). Based on a single MIFE measurement, the estimated heritability was relatively lower, being 0.73 from ANOVA of parents and F_1s, 0.82 from diallel analysis of F_1s and 0.80 from diallel analysis of F_2s. Using the average values from around 10 measurements, the heritability of the trait increased dramatically, being 0.97, 0.98 and 0.97 respectively from ANOVA of parents and F_1s, diallel analysis of F_1s and diallel analysis of F_2s.

Table 6.4. Heritability of salt tolerance based on NaCl-induced K^+ flux.

	h^2_B	h^2_N
Estimated from Table 6.2 based on single seedling	0.73	–
Estimated from Table 6.2 based on average value	0.97	–
Estimated from F_1 diallel analysis based on single seedling	0.82	0.76
Estimated from F_1 diallel analysis based on average value	0.98	0.91
Estimated from F_2 diallel analysis based on single seedling	0.8	0.64
Estimated from F_2 diallel analysis based on average value	0.97	0.79

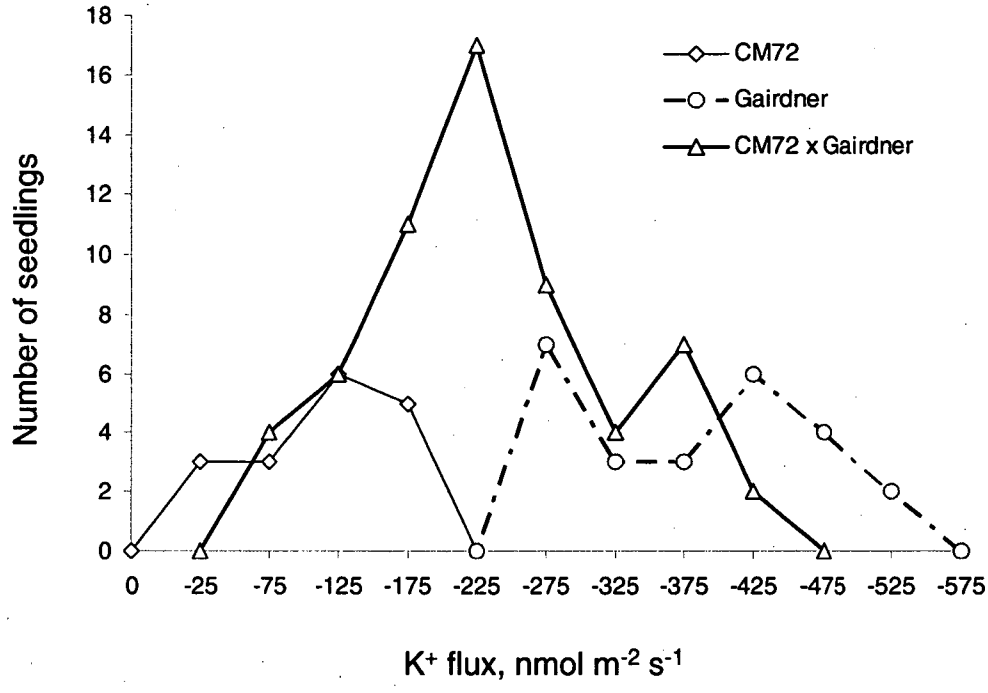

Figure 6.5. Distribution of root K^+ flux after salt treatment for a typical low K^+ efflux (CM72) × high K^+ efflux (Gairdner) F_2 population compared to the parents.

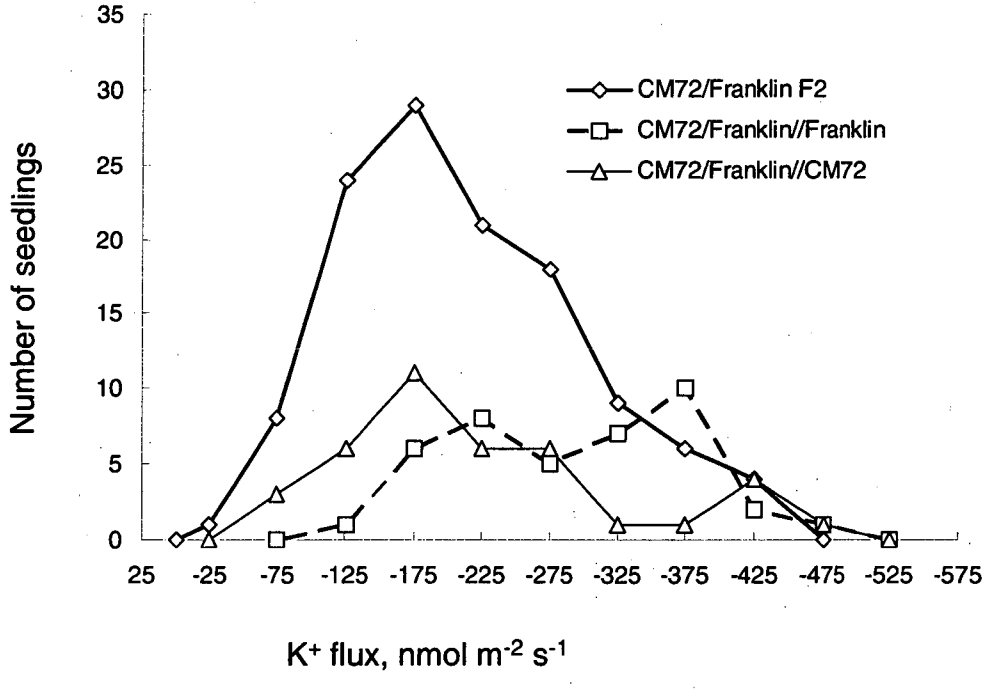

Figure 6.6. Distribution of root K^+ flux after salt treatment for F_2 and back crosses between CM72 × Franklin. The mean values of the parents and F_1 are indicated by arrows.

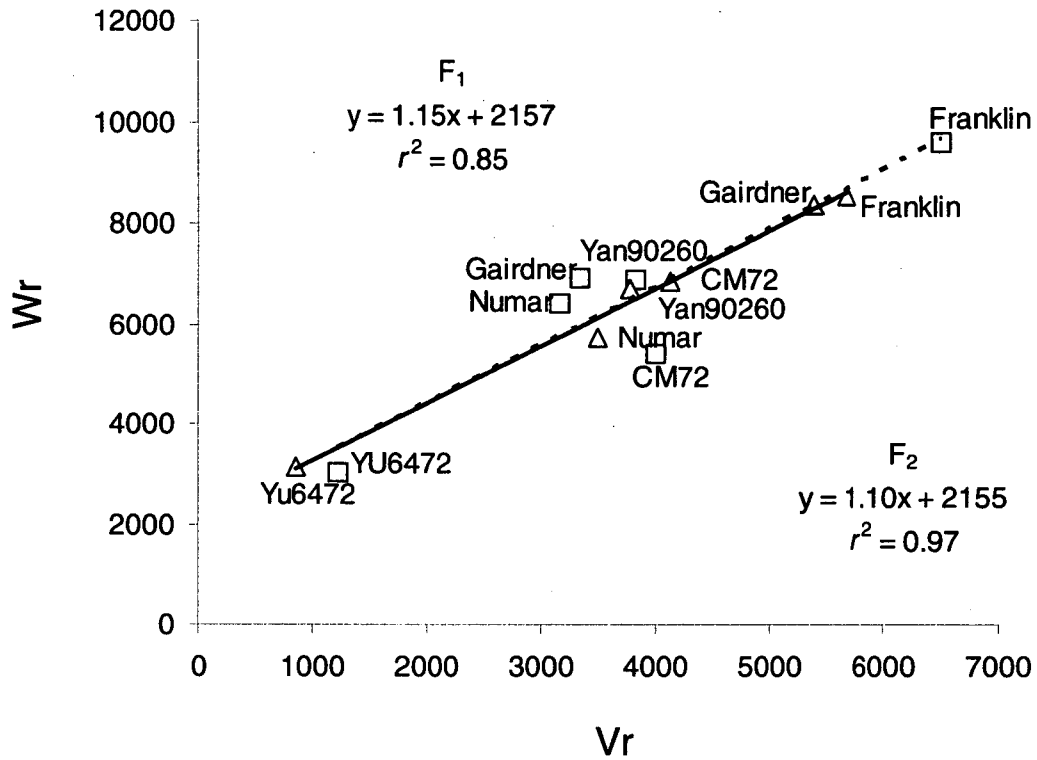

Figure 6.7. The Wr/Vr for NaCl-induced root K^+ flux measured from mature region of 3-d old barley roots after 1 h pretreatment in 80 mM NaCl (F_1: hollow square and dashed line; F_2: hollow triangle and solid line).

6.4. Discussion

6.4.1. Methods for screening salt tolerance

The complexity of salt tolerance, and difficulties in testing it for screening purposes in large populations, has led to the quest for 'rapid screening techniques' or 'physiological markers' as screening tools for salt tolerance (Shannon 1979). Any test has to meet several criteria in addition to an adequate predictive value. The method has to be relatively easy and simple to use, and allow large numbers of plants to be tested at low cost. A large number of screening methods have been reported, i.e. ranking of plants according to growth rate or yield (Greenway 1962), plant survival at high salinity (Sayed 1985), germination rate (von Well and Fossey 1998, Tajbakhsh et

al. 2006), leaf or root elongation rate (Cramer and Quarrie 2002), leaf injury and reduction of P_n (James et al. 2002), damage to the photosynthetic apparatus (Krishnaraj et al. 1993), Na^+ exclusion (García et al. 1995), Cl^- exclusion (Rogers and Noble 1992) and K^+ /Na^+ discrimination (Asch et al. 2000). Previously, a strong correlation was found between the NaCl-induced root K^+ flux, measured by the MIFE technique, and salt tolerance in seven barley cultivars (Chen et al. 2005). Further work has been extended to 70 different varieties and significant correlations ($r^2 = 0.6$) were found between K^+ flux measurements and relative grain yield under salt stress (Chen et al. 2007). In this experiment, the relative yields of the selected six parents were significantly correlated with NaCl-induced root K^+ flux (Figure 6.1), confirming the reliability of using the MIFE technique as a screening tool. Thus further genetic studies were conducted using K^+ flux measurements as an indication of salt tolerance.

6.4.2. Genetics of salt tolerance

Several previous reports failed to find any non-allelic gene interactions (Koval and Koval 1996; Mano and Takeda 1997a) but Maddur (1977) indicated that non-allelic gene interaction was important during early growth. In the current diallel study, the regression coefficient of Wr on Vr is slightly higher than 1 (significant for F_2 population), indicating the existence of non-allelic genes. Thus the additive-dominance model is not fully adequate to account for the behaviour of salinity tolerance involving these varieties. A further genetic model test was also conducted with the *A*, *B*, and *C* scaling test and joint scaling test using six generations (P_1, P_2, F_1, F_2, BC_1 and BC_2) from crosses of CM72/Gairdner and CM72/Franklin. Both tests indicated the inadequacy of the additive-dominance model. As discussed later in this chapter, the results of the test may be affected by less vigour of F_1, BC_1 and BC_2 seeds, which led to the generally greater K^+ efflux. Similar results were found in wheat by Woodend and Glass (1993) on the significance of additive, dominance and epistatic gene effects for the potassium utilisation efficiency. However, diallel analysis showed that salt tolerance was mainly controlled by additive effects even though dominant and epistatic effects can also contribute to the tolerance. In tomato, Foolad (1997) and Foolad and Jones (1991) also reported that more than 90% of the genetic variation among generations was due to additive genetic effects and that

dominance and non-allelic interaction effects were minimal.

Distribution of F_1, F_2 and backcross populations showed partial dominance for tolerance, which is different from the results reported by Mano and Takeda (1997a), who indicated that barley salt tolerance at germination was mainly controlled by recessive genes, and of Gregorio and Senadhira (1993) who reported overdominance effects for K^+/Na^+ discrimination in rice. The continuous distribution of salt tolerance in F_2 populations of crosses between salt-tolerant and -susceptible varieties indicated that salt tolerance was likely to be controlled by several genes. This is hardly surprising, as about 5% of the entire plant genome (data for *Arabidopsis*) is involved cation transporters (Mäser et al. 2002b). Just for K^+ transport, 75 genes from seven different families are known (Véry and Sentenac 2002; Shabala 2003). Previous electrophysiological studies suggested that several of them (particularly, KORCs and NSCCs) might mediate NaCl-induced K^+ efflux from plant tissues (Shabala et al. 2003, 2005a, b, 2006; Cuin and Shabala 2005, 2006). This is not likely to be a full list. My further studies will be focusing on the molecular identity of the candidate K^+ channels and transporters which might be responsible for this NaCl-induced K^+ efflux (Chapter 7).

6.4.3. Heritability of K^+ flux

Intensive genetic and molecular-marker studies have been carried out on K^+, Na^+, Cl^- uptake, and K^+/Na^+ ratio in some cereal species (Gregorio and Senadhira 1993; García et al. 1997; Koyama et al. 2001; Lin et al. 2004). Reports on diallel analyses have indicated significant additive and dominance genetic effects and a high level of heritability values for rice salt tolerance (Moeljopawiro and Ikehashi 1981). Noble et al. (1984) reported that Cl^- exclusion was a heritable character and a useful selection criterion in breeding for improved salt tolerance in lucerne. It was also reported that the inherent genetic capabilities of tomato variety PI174263 to maintain high tissue Ca^{2+} levels and to exclude Na^+ from the shoot were essential features of salt stress tolerance and that these features were highly heritable (Foolad and Jones 1991). High h^2_N estimates were observed for Ca^{2+}, K^+, Na^+, K^+/Na^+, and Ca^{2+}/Na^+ indicating the prime importance of additive effects in their genetic control (Rezai and Saeidi 2005). There are no reports, however, on the heritability of NaCl-induced K^+ efflux as a

physiological trait for salt tolerance. Such a gap is filled in this study. A very high heritability was found for salt tolerance when using NaCl-induced root K^+ flux as a selection criterion. Moreover, the heritability estimated from different approaches was very similar. The broad sense heritability (h^2_B) was over 0.7 based on single K^+ flux measurements. However, when the estimations were based on the average value of around 10 K^+ flux measurements, the heritability was increased dramatically to more than 0.95 (Table 6.4). Since NaCl-induced root K^+ flux was mainly controlled by additive effects as discussed above, high h^2_N was also found. The extremely high heritability of NaCl-induced root K^+ flux may also be due to the relatively small environmental effects on the MIFE measurement, compared to the many factors affecting field experiments.

6.4.4. Selecting for salt tolerance using the MIFE technique

Despite being skill-demanding, the MIFE technique is a fast screening tool for salt tolerance (average 40 plants per day) and the NaCl-induced K^+ flux measured by the MIFE technique was highly correlated with relative grain yield, shoot biomass, plant height, P_n, survival rate, and seed weight measured in glasshouse experiments (Chen et al. 2007). However, as discussed in Chapter 4, many factors can affect the MIFE measurement, including seedling age, root length, healthiness of the seedling etc. In the current experiment, consistent selection was made of uniform seeds with similar length of root for measurement, but the unavoidable disadvantage of F_1 and backcross seeds may still lead to some inaccurate estimation of some gene effects. During the experiments, lower germination and root growth rate were also observed for F_1 and backcross seeds, which may contribute to the lower average level of dominance estimated from F_1 ($\sqrt{H/D} = 0.28$) comparing that estimated from F_2 ($\sqrt{H/D} = 0.50$). More studies will be conducted on the factors affecting MIFE measurement to set up more reliable testing conditions. Since the average value provides much better estimation of salt tolerance, this technique will work better in homozygous populations such as doubled haploid lines.

Chapter 7. Root Plasma Membrane Transporters Controlling K^+/Na^+ Homeostasis in Salt Stressed Barley

7.1. Introduction

Intracellular K^+/Na^+ homeostasis is crucial for cell metabolism and is considered to be a key component of salinity tolerance in plants (Niu et al. 1995; Maathuis and Amtmann 1999; Shabala 2000; Hasegawa et al. 2000; Tester and Davenport 2003; Volkov et al. 2004; Chen et al. 2007). In order to maintain an optimal intracellular K^+/Na^+ ratio under saline conditions, accumulation of excessive amounts of Na^+ in the cytosol should be prevented, along with retention of physiological concentrations of cytosolic K^+. However, the understanding of how this is achieved is rather limited.

At the cellular level, maintenance of low cytosolic Na^+ may be achieved through several major strategies. One is to restrict unidirectional Na^+ uptake by roots (which is mediated mostly by NSCC: Davenport and Tester 2000; Demidchik and Tester 2002; Essah et al. 2003; Demidchik and Maathuis 2007). Another is active Na^+ extrusion from the cytosol to the external medium (mediated by PM-located Na^+/H^+ antiporters: Blumwald et al. 2000; Shi et al. 2002; Shabala et al. 2005a), and vacuolar compartmentation of Na^+ (via tonoplast-located Na^+/H^+ antiporters; Apse et al. 1999; Blumwald 2000; Zhang and Blumwald 2001). The last also contributes to cell osmotic adjustment (providing a lower cellular osmotic potential under hypertonic conditions of salt stress). At the whole-plant level, prevention of Na^+ transport to the shoot (Tester and Davenport 2003), and perhaps also recirculation of Na^+ back to the roots through the phloem (Lohaus et al. 2000; Nublat et al. 2001; Berthomieu et al. 2003) appear to be crucial for salinity tolerance. Most glycophytes have a poor ability to exclude salt (Niu et al. 1995; Munns 2002) and there is apparently a strong correlation between Na^+ exclusion and salinity tolerance in many species (Tester and Davenport 2003; Munns 2005).

The high cytosolic K^+/Na^+ ratio may also be achieved by efficient cytosolic K^+ homeostasis. Under saline conditions, the PM is strongly depolarised (by 60-80 mV; Shabala et al. 2003, 2005, 2006; Cuin and Shabala 2006). Although this reduces the

electrochemical driving force for Na^+ uptake, the more important effect of the depolarisation is to cause a drastic K^+ efflux from both root (Chen et al. 2005; Cuin and Shabala 2005) and mesophyll (Shabala 2000; Shabala et al. 2006a) cells, substantially reducing the cytosolic K^+ pools (Carden et al. 2003; Cuin et al. 2003; Shabala et al. 2006a) and compromising the metabolic competence of the cell. Increased uptake of K^+ is difficult to attain under saline conditions (due to the direct competition from Na^+ for K^+-binding sites on transport systems and due to a reduced electrochemical potential difference for passive K^+ uptake). Hence prevention of K^+ loss from cells appears to be crucial for maintaining cytosolic K^+ concentration.

Strong correlations have been reported (Chapters 4 and 5) between the ability of young barley seedlings to restrict NaCl-induced K^+ release and the salinity tolerance of mature plants, as measured by various physiological parameters (Chen et al. 2005, 2007). Genetic analysis has suggested that barley salinity tolerance, based on NaCl-induced K^+ efflux, is under polygenic control – mainly by additive genes with relatively smaller dominant and epistatic effects (Chapter 6). However, the specific ion transporters determining differential salt-sensitivity among genotypes, and the control modes of these transporters, were not investigated.

This issue was addressed in this study. A range of biophysical measurements (membrane potential, non-invasive MIFE ion flux, patch-clamp and radiotracer) and physiological and biochemical assays were applied to several barley cultivars contrasting in their salinity tolerance, as in Chapter 4. Results show that the superior ability of salt-tolerant cultivars to retain K^+ is determined by several factors. They are consistent with the idea of the cytosolic K^+/Na^+ ratio being a key determinant of plant salinity tolerance, and suggest multiple pathways of controlling that important feature in salt-tolerant plants.

7.2. Materials and methods

7.2.1. Plant materials and growth conditions

Six barley genotypes (three salt-tolerant - CM72, Numar and ZUG293; and three salt-sensitive - Franklin, Gairdner and ZUG403) were previously described in Chapter 3. Hydroponically grown 3-d old barley seedlings were used for all

electrophysiological and ion depletion experiments. For the H^+-ATPase assay, seeds were germinated on wet filter paper in Petri dishes, then grown for 7 d in the dark in vermiculite. In both cases, 0.5 mM KCl and 0.1 mM $CaCl_2$ was used as a bath or watering solution. For leaf sap Na^+ analysis, barley plants were grown semi-hydroponically in a glasshouse. Growth medium and condition were previously described in Chapter 4. 320 mM NaCl treatment, added to three-week old plants, was reached by starting at 80 mM NaCl with a 40 mM daily increment. Salinity treatment lasted for four weeks.

7.2.2. Ion depletion experiments

Net Na^+ uptake and K^+ loss from barley roots were studied in depletion experiments. Roots of ten intact 3-d old seedlings were immersed in a plastic vial with 10 ml saline solution (80 mM NaCl, 0.5 mM KCl, and 0.1 mM $CaCl_2$) and aerated with an aquarium air pump. Seedlings were kept at 25°C in the dark for 24 h, then roots were blotted dry, cut and weighed. Na^+ and K^+ concentrations in the remaining solution were determined using flame photometry, and net Na^+ uptake and K^+ loss were calculated on a fresh weight basis. Two independent experiments were conducted with three replicates per cultivar in each experiment.

7.2.3. Leaf sap Na^+ concentration

Measurement of tissue Na^+ concentration was described by Cuin and Shabala (2005). Flag leaves were collected into 1.5-ml microcentrifuge tubes and immediately frozen by liquid nitrogen. A basal opening in the tube allows cell sap but not tissue fragments to pass through to a collection tube. The sample was then thawed and spun for 3 min at 11,000×g in a microcentrifuge. The collected sample was measured for its Na^+ concentration (in mM) using a flame photometer.

7.2.4. K^+ flux measurements

Steady-state K^+ flux of 6 barley cultivars was measured 1 h after the imposition of salt stress (80 mM NaCl, 0.5 mM KCl, and either 0.1 or 1 mM $CaCl_2$). For pharmacological experiments, seedlings were pre-treated in the low Ca^{2+} (0.1 mM) saline solution for 1 h in 5 ml of saline solution as above. After K^+ fluxes had been measured for 30 min, K^+ channel blockers (either 20 mM tetraethylammonium chloride (TEA^+), or 50 μM $GdCl_3$) were added to the solution and K^+ fluxes recorded for another 30 min. See 3.3.2 for more details.

7.2.5. Na^+ influx measurements

Na^+ influx was measured using $^{22}Na^+$ radiotracer essentially as described by Essah et al. (2003). Entire root systems of 3-d old seedlings were excised from the shoot (to eliminate potential complications arising from transpiration), and used for experiments immediately after blotting with tissue paper. To determine the steady-state Na^+ influx (avoiding the effects of sudden salt-shock), seedlings were pre-treated for 24 h in the saline solution. Tracer influx was measured from 10 ml of unbuffered saline solution containing approximately 40 kBq mL^{-1} of $^{22}Na^+$, at two Ca^{2+} concentrations (0.1 mM and 10 mM) and over five durations (1, 2, 5, 10, and 20 min). On average, ten roots per treatment were measured for each duration. At the end of the influx period, roots were blotted dry and transferred into 500 ml of ice-cold 80 mM NaCl plus 10 mM $CaCl_2$ for two successive rinses of 2 min and then 3 min to displace any apoplastic $^{22}Na^+$. All solutions were stirred on gently moving shakers at 45 rpm. Roots were blotted gently, rapidly weighed, and transferred to plastic vials containing 2.5 ml scintillation cocktail (Optiphase Hisafe, Fisher Chemicals, Loughborough, UK). Samples were counted with a liquid scintillation counter (Beckman coulter LS6500, Fullerton, CA, USA).

7.2.6. Membrane potential measurements

Conventional KCl-filled Ag/AgCl microelectrodes (Shabala and Lew, 2002; Cuin and

Shabala, 2005) with a tip diameter 0.5 μm were used with the MIFE electrometer to measure E_m from epidermal cells in the root mature zone. Following cell penetration, E_m was recorded for 2 min and then an equal quantity of the standard solution having 160 mM NaCl was added, giving the required 80 mM NaCl concentration after mixing. Measurements continued for at least 20 min after addition of the saline solution. In steady-state experiments, measurements were taken in control roots and in roots 20 min after 80 mM NaCl treatment. At least five individual plants for both control and treated roots were determined, with up to three measurements from each individual root.

7.2.7. Protoplast isolation for patch-clamping

An effective protocol for the quick isolation of root epidermal protoplasts was developed by modifying the previously described protocols used for mesophyll protoplasts (Demidchik and Tester 2002; Shabala et al. 2006a). The advantages of the method are: (1) short preparation time (~30 min altogether); (2) minimum contamination of the measuring chamber by cell debris; (3) direct release of the fresh protoplasts into the measuring chamber, without any centrifugation step (hence with minimal disturbance to protoplasts); and (4) a fresh isolation for each patch-clamp measurement (hence, higher success rate of gigaohm seal formation).

According to the protocol developed, a 3-d old barley seedling was removed from the growing container. Seminal roots were cut at about 5 mm below the seed, and their apical 7-10 mm were also cut and discarded. The remaining segments were cut into ~ 10 mm lengths and split longitudinally under a dissecting microscope. Split root segments were placed into 3 ml of the enzyme solution containing 2% [w/v] cellulose (Yakult Honsha, Tokyo, Japan), 1.2% [w/v] cellulysin (Biosciences Inc., San Diego, CA, USA), 0.1% [w/v] pectolyase, 0.1% [w/v] bovine serum albumin, 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, pH 5.7 adjusted with 2 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). All chemicals and reagents were purchased from Sigma unless specified otherwise. The osmolality of the enzyme solution was adjusted to 760–800 mOsM with mannitol. After 20–25 min of incubation in the enzyme solution (in the dark at 30°C; agitated on a 90 rpm rotary shaker), root segments were transferred to the so-called "wash solution" (as above,

minus enzymes) and thoroughly washed for another 2 min. Segments were then transferred into the measuring chamber filled with "release" solution (10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$; 2 mM MES, pH 5.7; osmolality 380 mOsM). By gently shaking the plasmolysed and digested root tissue, protoplasts were released into the measuring chamber. Root tissues were removed from the solution, and the chamber was then perfused with the bath solution used for patch-clamp experiments (see next section), removing all protoplasts that were not attached to the bottom of the measuring chamber.

The above protocol provides protoplasts from entire roots. I wished to use epidermal protoplasts for optimum match with the E_m and flux studies. To the best of my knowledge, no suitable tissue-specific staining technique is available for barley to provide specific tissue identification. As a result, protoplast selection for patch-clamp experiments was based on the protoplast diameter (~ 20 μm), which was indicative of an epidermal origin. To justify this choice, separate experiments were undertaken. Protoplasts were isolated from (1) the whole root; (2) isolated root epidermis; (3) stele; and (4) cortex. Overall, over 5000 protoplasts were measured (Table 7.1). Results showed that cortical protoplasts were twice as large as those isolated from epidermal or stele tissues, thus they could be easily distinguished and avoided in patch experiments. Accordingly, protoplasts isolated from the whole root showed a clear bimodal distribution in diameter (Figure 7.10). The average diameter of stele and epidermal protoplasts, however, was somewhat similar (Table 7.1) and close to the 20 μm size chosen (Figure 7.10 arrow) for patch experiments. However, the yield of stellar protoplasts was much lower (only 6% compared with epidermal ones), perhaps due to the lignification pattern of the stele making it almost inaccessible to enzymes during the digestion. Therefore, although the possibility can not be excluded that some protoplasts measured in this study originated from the xylem parenchyma, the proportion of them is low (~ 6%; Figure 7.10 and Table 7.1).

Table 7.1. Basic characteristics of protoplasts isolated from different parts of the mature region of barley roots.

Tissue type	*Mean diameter, μm*	*S.D.*	*Sample size*	*Number of isolations*	*Total number of protoplasts*
Entire root	21.4	6.2	212	4	1400
Epidermis	14.2	5.1	352	4	900
Stele	16.7	4.2	50	4	50
Cortex	33.3	8.2	509	3	3100

7.2.8. Patch-clamp experiments

Barley root protoplasts of 14 to 22 μm diameter were patch-clamped in the whole-cell mode. GΩ resistance seals were obtained in the bath solution containing (in mM): 1 to 2 $CaCl_2$, 5 KCl, 2 MES, pH 5.7, 500 mOsM adjusted with D-sorbitol. The basic pipette solution (PS) contained (in mM): 100 KCl, 2 $MgCl_2$, 0.8 $CaCl_2$ (100 nM free Ca^{2+}), 2 ethylene glycol tetraacetic acid (EGTA), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 adjusted with trishydroxymethylaminomethane (Tris) base. Osmolality of the PS was 560 mOsM.

Measurements were made using an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) in the conventional whole cell configuration as described by Shabala et al. (2006). Membrane potentials were clamped at -100 mV throughout the experiments, and voltage pulses were applied in 20 mV steps, from -160 mV to +80 or + 100 mV. Typical access resistance was 11–32 MΩ, and mean whole cell capacitance, 11.9 ± 0.8 and 11.0 ± 1.0 pF for Gairdner and CM72 protoplasts, respectively.

7.2.9. Isolation of plasma membranes for PM H^+-ATPase assay

Barley roots (5 to 12 g fresh weight) were rinsed with bathing solution or water to remove the vermiculite. Roots were then homogenised in 200 ml buffer (250 mM Tris-HCl pH 8.0, 300 mM sucrose, 25 mM ethylene diamine tetracetic acid (EDTA), 5 mM dithiothreitol (DTT), 5 mM ascorbate, 0.6% polyvinylpyrrolidone (PVP) and 1

mM phenylmethylsulfonyl fluoride (PMSF)) containing phosphatase inhibitors (25 mM NaF, 1 mM NaMo, 50 mM Na-pyrophosphate). Plasma membranes were isolated from the microsomal fraction (30,000 × g) by partitioning at 4 °C at an aqueous polymer two-phase system (9 *g* + 3 *g*) composed of 6.3% (w/w) dextran T500 (Amersham Biosciences, GE Healthcare, Copenhagen), 6.3% (w/w) polyethylene glycol (PEG) 1500 (Sigma, Copenhagen), 330 mM sucrose, 5 mM potassium phosphate pH 7.8, 3 mM KCl, 0.1 mM EDTA and 1 mM DTT (Palmgren 1990; Larsson et al. 1994). The final PM pellet was suspended in 330 mM sucrose, 5 mM potassium phosphate pH 7.8, 50 mM KCl, 5 mM EDTA.

7.2.9.1. Western blotting

Protein concentration was determined by Bradford assay using gamma-globulin as a reference. Proteins (20 μg/lane) were solubilised in SDS cocktail and subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose paper for immunostaining. Antiserum #759 against the C-terminal domain of the PM H^+-ATPase was employed (dilution 1:4000) (Pardo and Serrano 1989).

7.2.9.2. ATPase assay

ATP hydrolytic activity was measured essentially as described by Regenberg et al. (1995). The assay medium (20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 8 mM $MgSO_4$, 50 mM KNO_3, 5 mM $NaNO_3$, 250 μM NaMo; pH adjusted to 6.5 or 7.0 with KOH) included 3 mM ATP and 0.02% Brij-58. The plasma membranes were pre-incubated for 10 min with 0.02% Brij-58 in order to obtain inside-out vesicles. The reaction was initiated by the addition of 2 μg of barley plasma membranes to the assay medium.

7.3. Results

Barley varieties contrasting in their salt tolerance were employed throughout this study. These clustered into two distinct groups: (1) salt-tolerant –Numar and ZUG293 (2) salt-sensitive – Gairdner and Franklin (as illustrated in Figure 7.1A). When

measured at the 3-d old stage, salt-tolerant genotypes showed a significant, 3-fold higher ($P < 0.01$) ability to retain K^+ in the root by minimizing NaCl-induced K^+ efflux from epidermal cells (Figure 7.1B). The magnitude of NaCl-induced K^+ loss showed a high correlation with salinity tolerance using conventional physiological and agronomical indices (Chapter 5, Chen et al. 2007). Under severe (320 mM) salinity stress, salt-sensitive genotypes failed to produce any seed, while salt-tolerant ones attained approximately 15% the grain yield of the control (Chapter 5, Chen et al. 2007). NaCl-induced K^+ loss was significantly ameliorated by the addition of 1 mM Ca^{2+} (a concentration typically found in a soil solution; Tisdale et al. 1993), in both salt-tolerant and salt-sensitive genotypes (Figure 7.1C). However, regardless of the Ca^{2+} concentration used, salt-tolerant varieties showed much better K^+ retention ability compared with salt-sensitive ones (Figure 7.1C). Moreover, using low Ca^{2+} levels increased the resolution of the method, resulting in a larger K^+ flux difference between contrasting varieties. This methodological advantage was kept in mind while conducting pharmacological and membrane potential measurements.

7.3.1. TEA^+-sensitive K^+ channels determine the difference in NaCl-induced K^+ efflux between contrasting genotypes

Two channel blockers, TEA^+ (K^+-selective channel blocker) and $GdCl_3$ (non-specific cation channel blocker) were used in pharmacological experiments. Consistent with previous results, pre-treatment with 80 mM NaCl for 1 h resulted in a significant difference in steady net K^+ flux, with a 3-fold larger K^+ loss from salt-sensitive Gairdner compared with salt-tolerant ZUG293 (Figure 7.2). Applying 20 mM TEA^+ significantly (~ 80%) reduced the K^+ loss from roots of Gairdner, but had a much smaller effect on the K^+ loss from salt-treated ZUG293. As a result, no significant difference in the magnitude of K^+ flux was observed between the contrasting varieties after TEA^+ treatment for 30 min. This suggests that the TEA^+-sensitive population of K^+ efflux channels are the main contributors towards NaCl-induced K^+ loss in salt-sensitive Gairdner, but this component has little contribution in salt-tolerant ZUG293. No significant effect of 50 μM Gd^{3+} treatment was observed for either cultivar after 1 h salt treatment (Figure 7.2B).

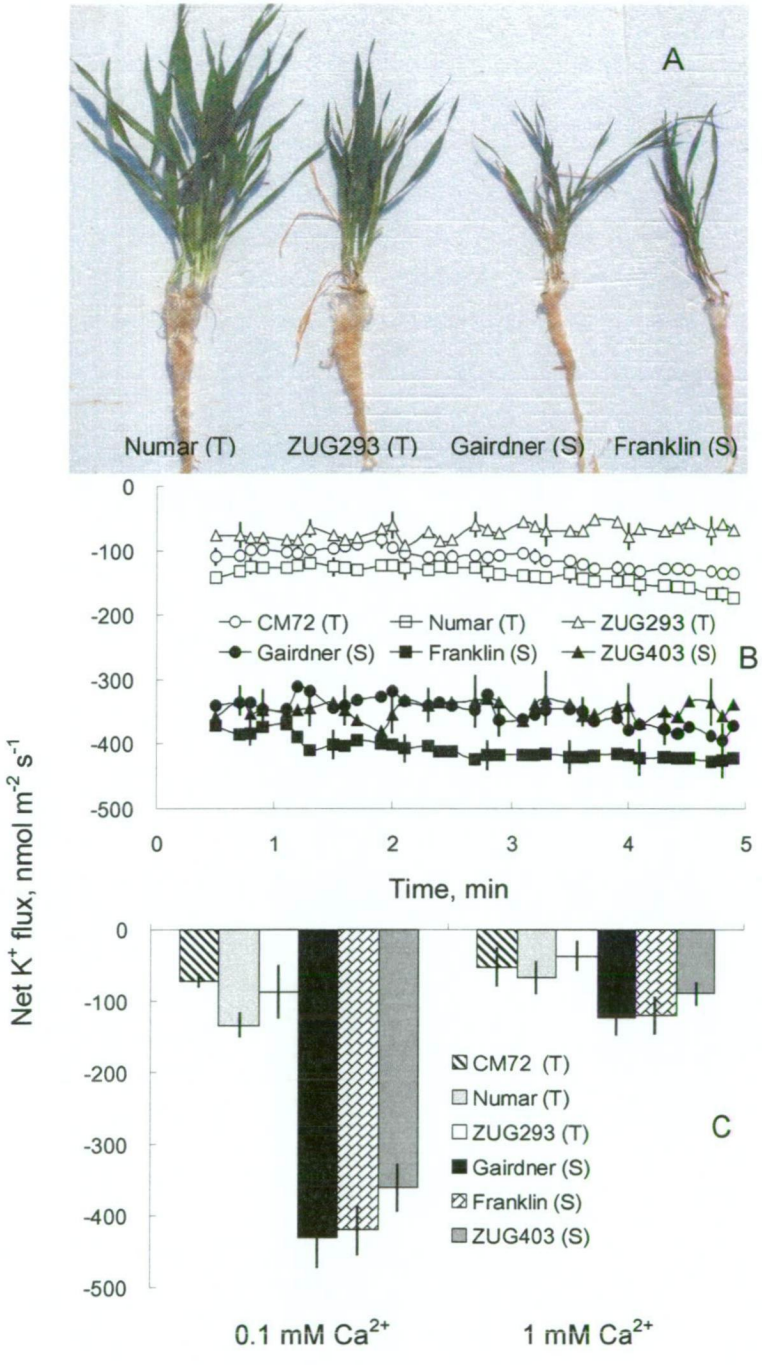

Figure 7.1. (A) – contrasting barley genotypes grown under 320 mM NaCl for four weeks in the glasshouse experiment. Salt-tolerant (T) and salt-sensitive (S) varieties are easily distinguished. (B) – steady-state net K^+ fluxes (inward positive) and (C) – effects of different external Ca^{2+} (0.1 and 1 mM) on NaCl-induced K^+ flux measured from 3-d old roots of barley genotypes contrasting in their salinity tolerance after 1 h of 80 mM NaCl treatment. Results in panel C are averaged over 15 min of K^+ flux measurement. Means ± SE (n = 7–10).

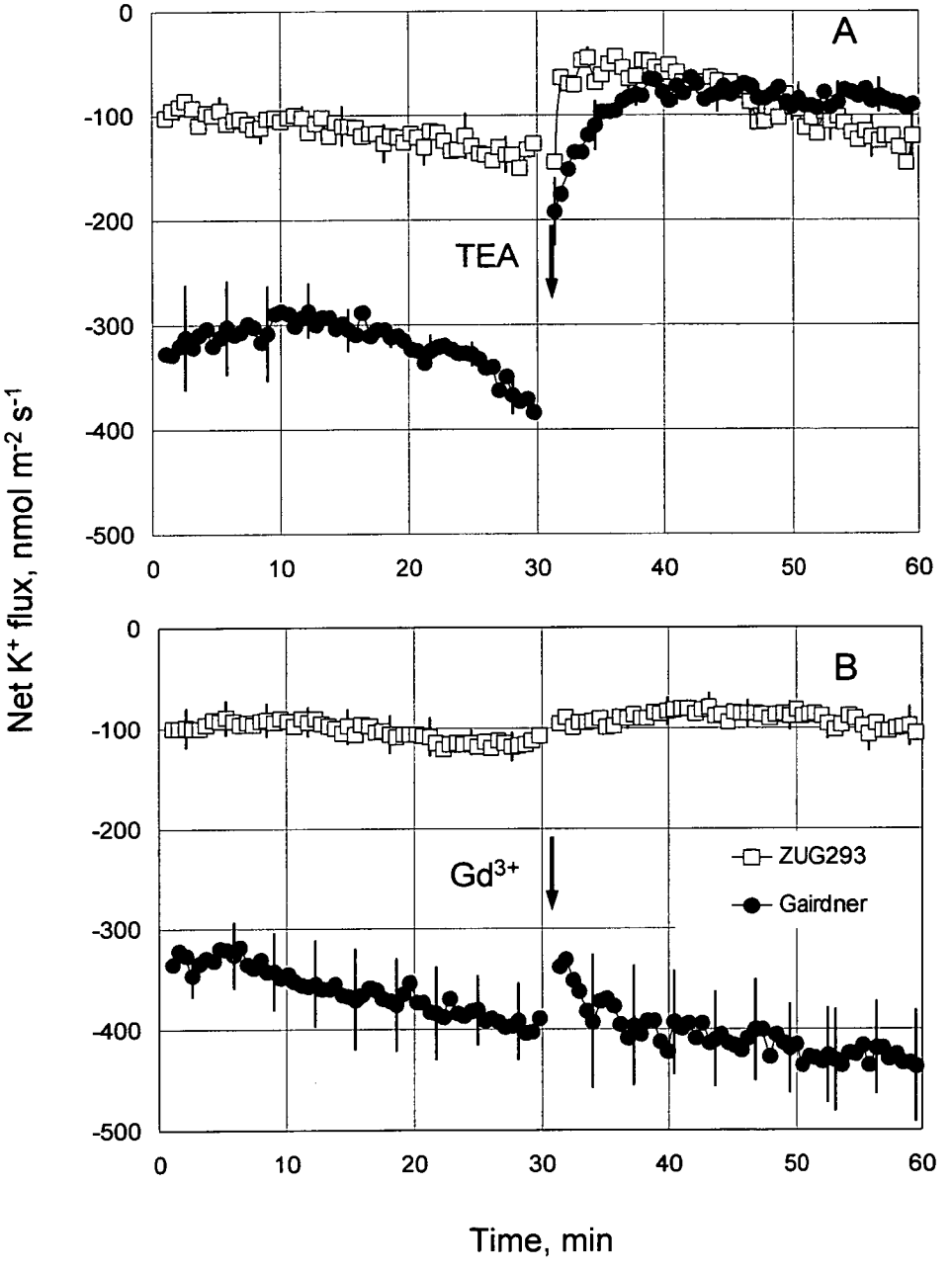

Figure 7.2. Pharmacology of K^+ flux responses. Net K^+ fluxes were measured in response to 20 mM TEA^+ (A) or 50 μM Gd^{3+} (B), applied at arrow, from roots of two contrasting barley genotypes (salt-tolerant ZUG293; salt-sensitive Gairdner) pre-incubated in 80 mM NaCl for 1 h. Means ± SE (n = 7–8).

7.3.2. Salt-tolerant genotypes have intrinsically higher H^+ pump activity and are capable of maintaining a more negative membrane potential

In plant cells, TEA^+-sensitive K^+ efflux channels are also voltage sensitive (Maathuis et al. 1997; Shabala et al. 2006a). Thus, the different K^+ retention ability of barley roots of contrasting genotypes might be related to a difference in their ability to maintain the E_m after the imposition of salt stress. The E_m of five genotypes was measured using conventional microelectrode impalement. Application of 80 mM NaCl caused an immediate and rapid depolarisation by about 70 mV (Figure 7.3A). Despite some recovery, the depolarised E_m was maintained, allowing comparison of the magnitude of E_m of all the cultivars before (2–4 min), and after (25–30 min), salt stress. This depolarisation was significantly ($P < 0.01$) greater in salt-sensitive than in salt-tolerant genotypes (Figure 7.3B) and strongly ($r^2 = 0.93$, $P < 0.01$) correlated with net K^+ flux. Notably, the E_m of salt-sensitive cultivars was ~ 10 mV more positive than the E_m of salt-tolerant varieties when measured under control conditions (Figure 7.3B).

This issue was addressed directly by measuring ATP hydrolytic activity from PM vesicles isolated from the microsomal fraction of roots (Figure 7.4A). The two salt-sensitive genotypes, Gairdner and ZUG403, had the lowest level of PM H^+-ATPase activity (5-fold lower than salt-tolerant CM72 and Numar). At pH 6.5 a strong correlation ($r^2 = 0.85$) between PM H^+-ATPase activity and NaCl-induced changes in E_m was found (Figure 7.4C). Western blot analysis demonstrated that the observed difference in PM H^+-ATPase activity could not be explained by a difference in enzyme level (Figure 7.4B) suggesting that the observed differences in H^+ pumping are the result of a post-translational modification of activity. This suggests that a higher specific PM H^+ pump activity is a characteristic of salt-tolerant varieties.

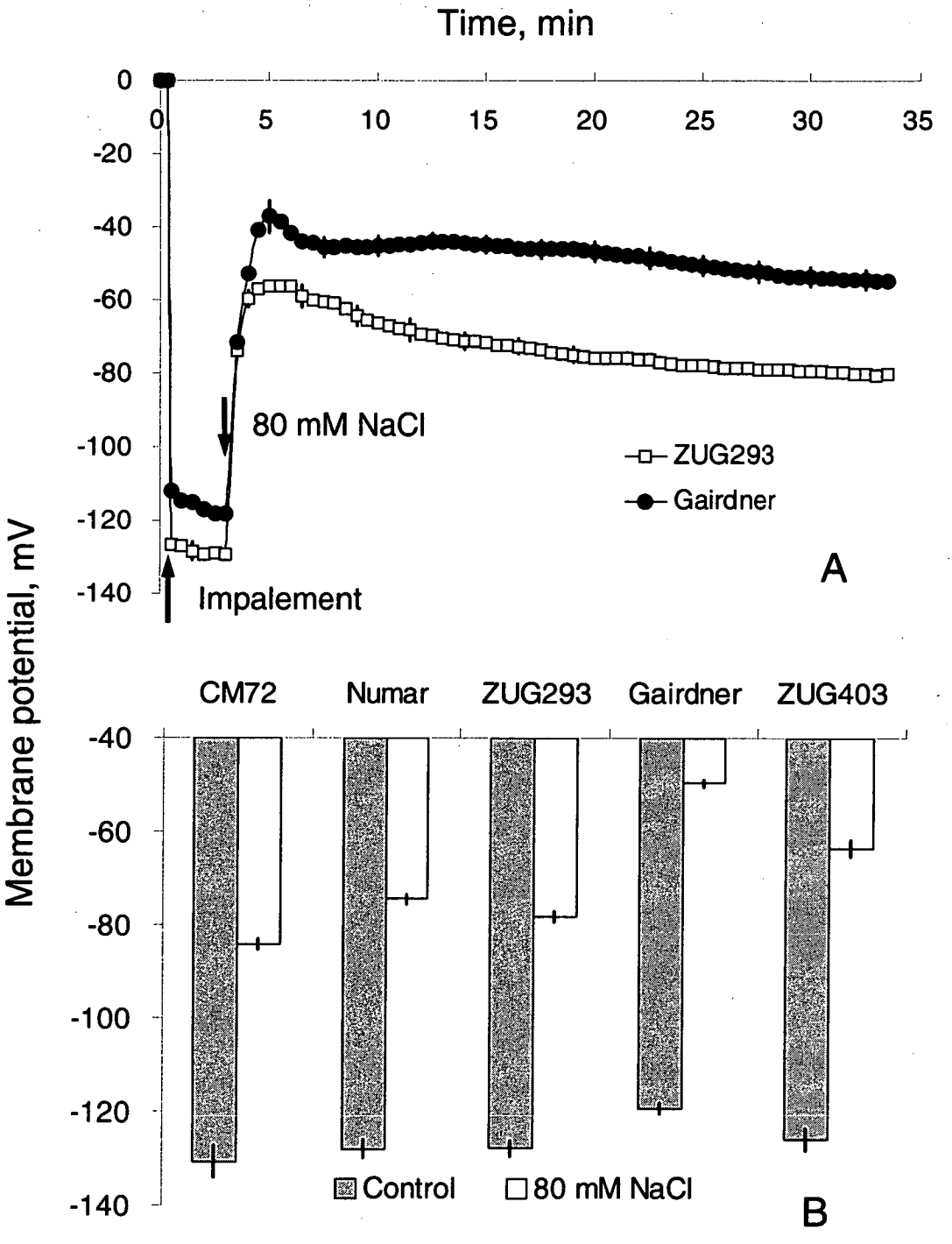

Figure 7.3. (A) – membrane potential of epidermal root cells of salt-tolerant ZUG293 and salt-sensitive Gairdner measured in response to 80 mM NaCl treatment (at arrow). Means ± SE (n = 6). (B) – steady-state E_m values in control (prior to NaCl treatment) and after 20 min root exposure to 80 mM NaCl. Means ± SE (n = 10).

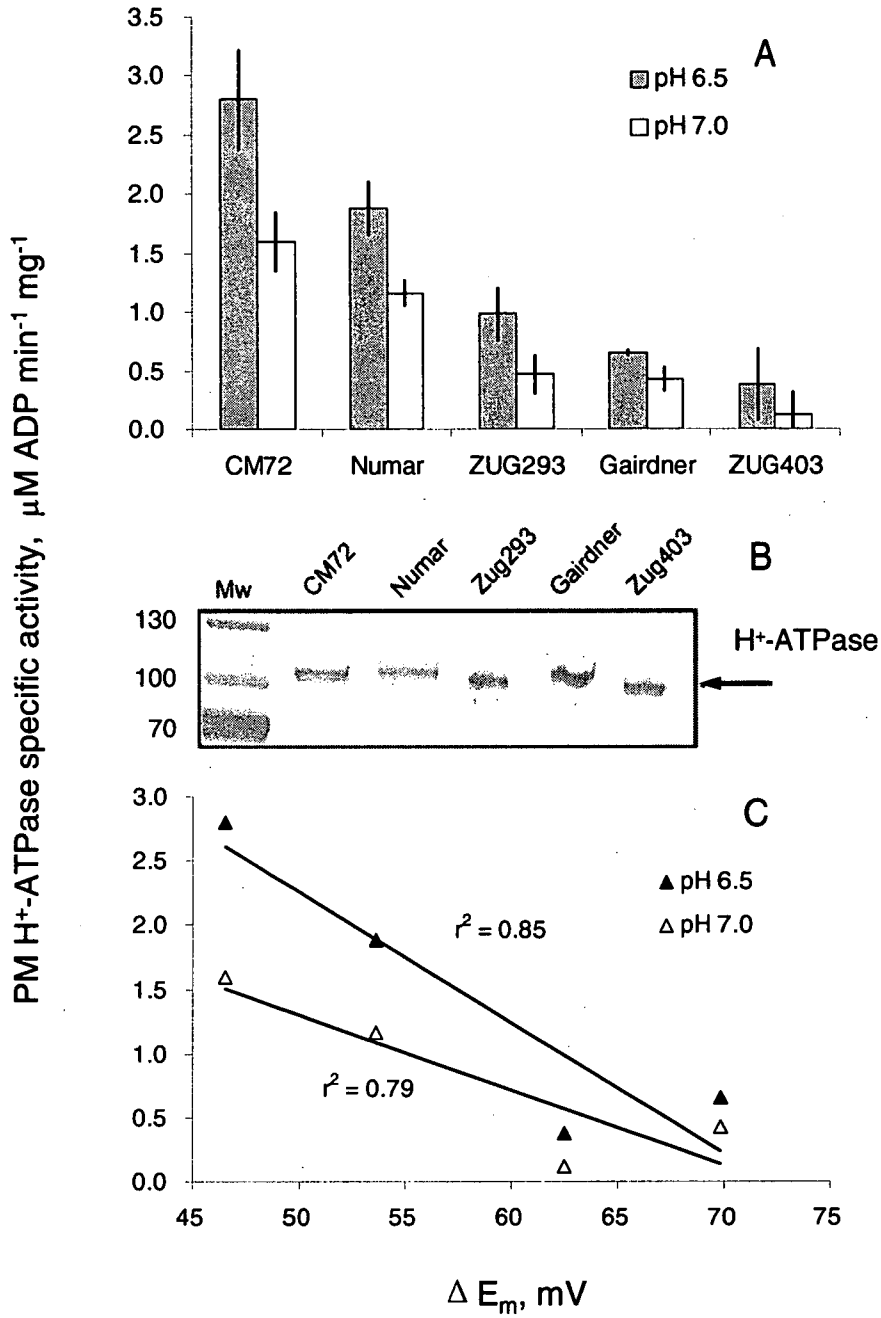

Figure 7.4. (A) – ATP hydrolytic activity of plasma membranes isolated from the microsomal fraction of roots of barley genotypes contrasting in salinity tolerance. Means ± SE (n = 6). The statistics are based on two independent PM preparations, and each of the preparations was tested three times with reproducible results. (B) –Western blot results demonstrated that the observed difference in the H^+-ATPase activity is not due to a difference in the enzyme level. (C) – correlation of ΔE_m and H^+-ATPase activity at 6.5 and 7 pH levels. Dr Fuglsang A. and Professor Palmgren M. at the University of Copenhagen obtained the results for this thesis.

7.3.3. Salt-tolerant genotypes accumulate less Na^+, but do not differ in unidirectional Na^+ uptake

A reduced Na^+ influx in salt-tolerant genotypes is another potential contributor to their better maintenance of membrane potential in saline conditions. I measured unidirectional $^{22}Na^+$ influx in response to sudden salt shock (Figure 7.5A) and after 24 h of salt treatment (Figure 7.5B). Rapid accumulation of $^{22}Na^+$ was measured in all genotypes upon addition of 80 mM NaCl, with $^{22}Na^+$ influx gradually decreasing during the first 20 min (Figure 7.5A), while a relatively steady $^{22}Na^+$ influx was observed after 24 h of NaCl pre-treatment (Figure 7.5B). However, no clear difference between contrasting cultivars was evident either immediately upon NaCl treatment, or after 24 h (Figure 7.5A, and B).

Solution depletion experiments showed that salt-tolerant genotypes were able not only to lose approximately 80% less K^+ (Figure 7.6A), but also significantly to reduce (by ~ 40%; $P < 0.05$) net root Na^+ uptake compared with salt-sensitive genotypes (Figure 7.6B). This implies that, given they have the same Na^+ unidirectional influx as salt-sensitive varieties, salt-tolerant genotypes have a higher capacity to extrude the Na^+ actively back to the external medium. This hypothesis was further tested by measuring Na^+ concentration in the flag leaf sap of each of six barley genotypes exposed to longer-term salt treatment (Figure 7.7). As expected, salinity stress resulted in a substantial (10 to 14 fold) increase in the leaf sap Na^+ concentration (Figure 7.7). Interestingly, salt-tolerant varieties showed relatively constant sap Na^+ levels, regardless of the duration of salt treatment (at around ~ 300 mM Na^+; Figure 7.7B). On the contrary, three salt-sensitive varieties showed a progressive accumulation of Na^+ in the flag leaf (Figure 7.7B). As a result, after 8 d of 320 mM NaCl treatment, salt-tolerant varieties had even slightly larger quantities of Na^+ in the leaf sap (308 ± 10 and 255 ± 10 mM; n = 12 for salt-tolerant and -sensitive group, respectively); significant at $P < 0.05$). Four weeks exposure to salt stress, however, resulted in salt-sensitive varieties accumulating 35% more leaf sap Na^+ than salt-tolerant ones (455 ± 17 and 337 ± 5 mM (n = 12), respectively; significant at $P < 0.05$; Figure 7.7B).

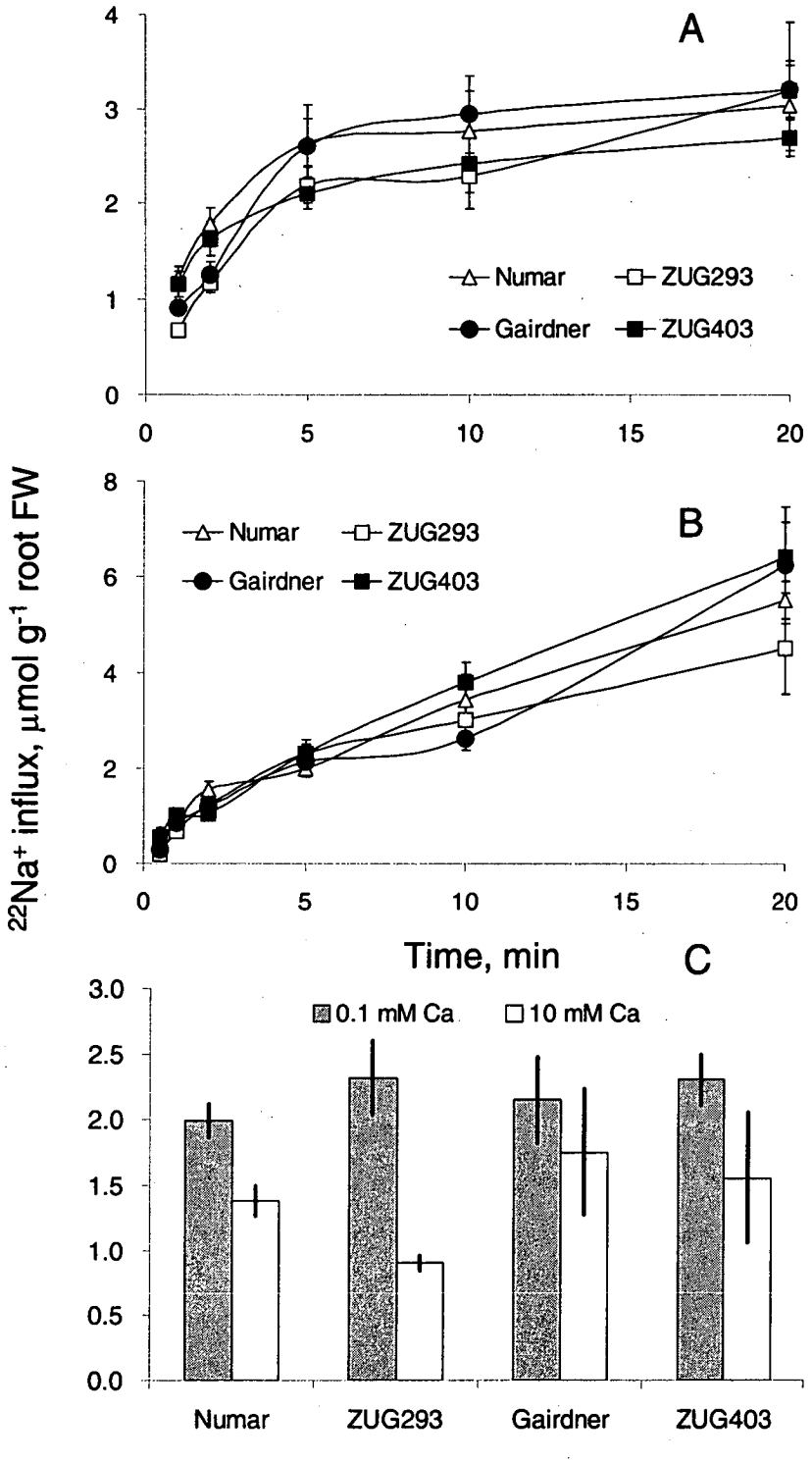

Figure 7.5. Unidirectional $^{22}Na^+$ uptake, at times up to 20 min, into excised roots of 4 barley cultivars contrasting in their salinity tolerance. (A) – immediately after 80 mM NaCl treatment; (B) – after 24 h incubation in 80 mM NaCl. (C) – for the immediate treatment, the $^{22}Na^+$ uptake during 5 min with two levels of external Ca^{2+} (0.1 as in A, and 10 mM). Means ± SE (n = 8–13).

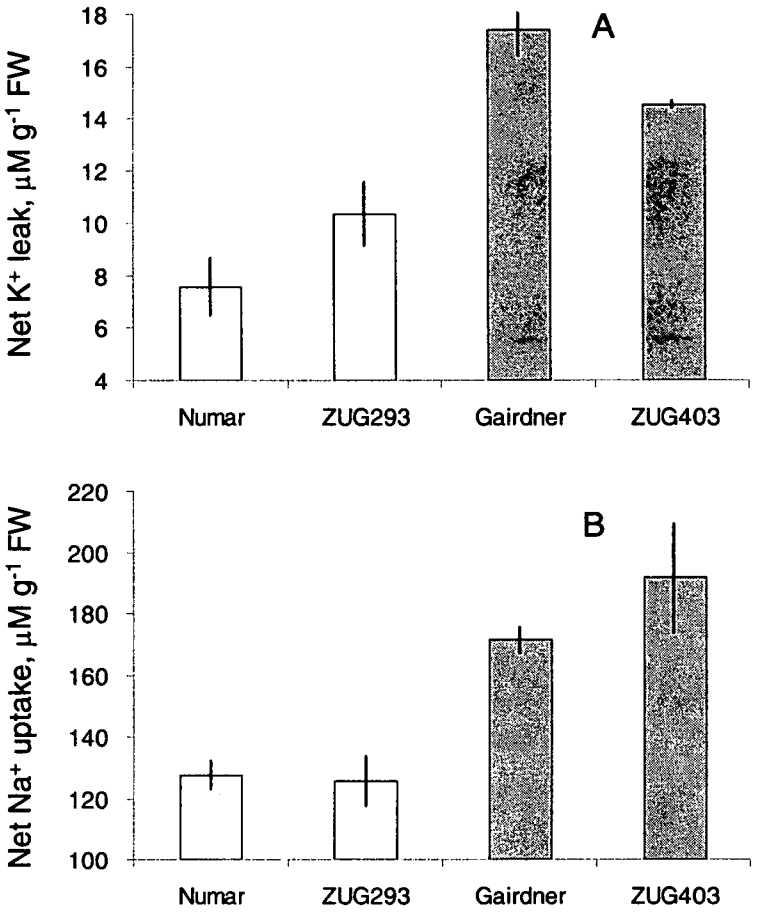

Figure 7.6. K^+ loss (A) and Na^+ uptake (B) by barley roots measured in depletion experiments. In each treatment, roots of ten 3-d old seedlings were immersed in 10 ml saline solution (80 mM NaCl; 0.5 mM KCl; 0.1 mM $CaCl_2$) in a plastic test tube and aerated for 24 h in the dark at 25°C. Two individual measurements were performed with three replicates for each genotype. Means ± SE (n = 6).

The above experiments were conducted under low (0.1 mM) external Ca^{2+} conditions. When $^{22}Na^+$ influx was measured with high (10 mM) external Ca^{2+}, the salt-tolerant genotypes Numar and ZUG293 showed a significant reduction in unidirectional $^{22}Na^+$ influx (on average ~ 46% of control values; $P < 0.05$; Figure 7.5C), whereas unidirectional $^{22}Na^+$ influx into roots of salt-sensitive genotypes (Gairdner and ZUG403) was much less affected by increased external Ca^{2+}. This suggests that Na^+ permeable transporters in the roots of salt-tolerant genotypes have a higher sensitivity to supplemental Ca^{2+}.

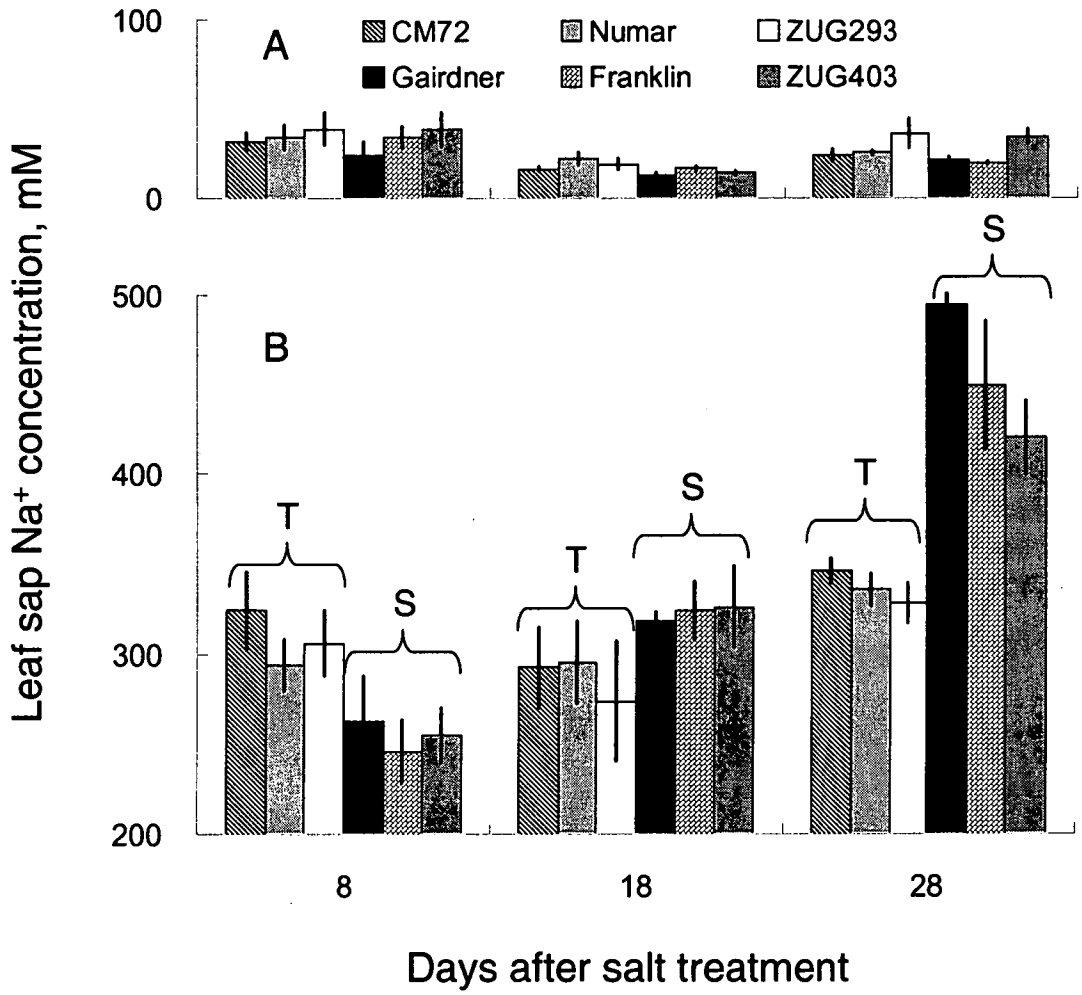

Figure 7.7. Leaf sap Na^+ concentrations of six barley genotypes in both control (A) and 320 mM NaCl treatment (B). Flag leaf samples of all cultivars were collected 8, 18, and 28 d after the imposition of salinity. Means ± SE (n = 4).

7.3.4. KOR-mediated currents in root epidermal protoplasts do not differ in salt-tolerant and -sensitive cultivars

Further electrophysiological characterisation of transport systems potentially involved in Na^+ and K^+ homeostasis in salinised barley roots was undertaken in a series of patch-clamp experiments. At least five cation currents were found, only one of which was sensitive to externally applied TEA^+ (result not shown) and was very similar to the KOR channel current previously described in barley xylem parenchyma (Wegner and Raschke 1994). In the ionic conditions used (5 mM external and 100 mM internal K^+), this current was activated at potentials positive of -40 mV with a characteristic time delay. The time course could be explained by Hodgkin-Huxley kinetics/ exponential power function, with *n* between 3 and 3.7 (results not shown). A typical recording of this K^+-selective outwardly rectifying (KOR) current is presented in Figure 7.8. The reversal potential of the KOR current was approximately -60 mV, close to E_k (K^+ equilibrium potential) which was -70 mV under these conditions. The deviation from the ideal K^+ selectivity probably reflects a limited Ca^{2+} permeability of this channel (Roberts and Tester 1997; Wegner and De Boer 1997).

Analysis of the occurrence (percentage of successful recordings in the total number of protoplasts recorded) and current densities of KOR channels for two contrasting genotypes, salt-sensitive Gairdner and salt-tolerant CM72, suggest that, despite having a slightly higher percentage of KOR channels in the salt-sensitive genotype (37% *vs* 30% of the total protoplast population studied, n = 70, 37 respectively; Figure 7.9A), the actual current density through KOR channels was slightly higher in the protoplasts from the salt-tolerant variety CM72 (Figure 7.9A). As a result, the overall K^+ current through KOR channels per protoplast was not significantly different between the contrasting varieties (Figure 7.9A). Also, no significant difference in KOR voltage-gating was found between contrasting genotypes (Figure 7.9B).

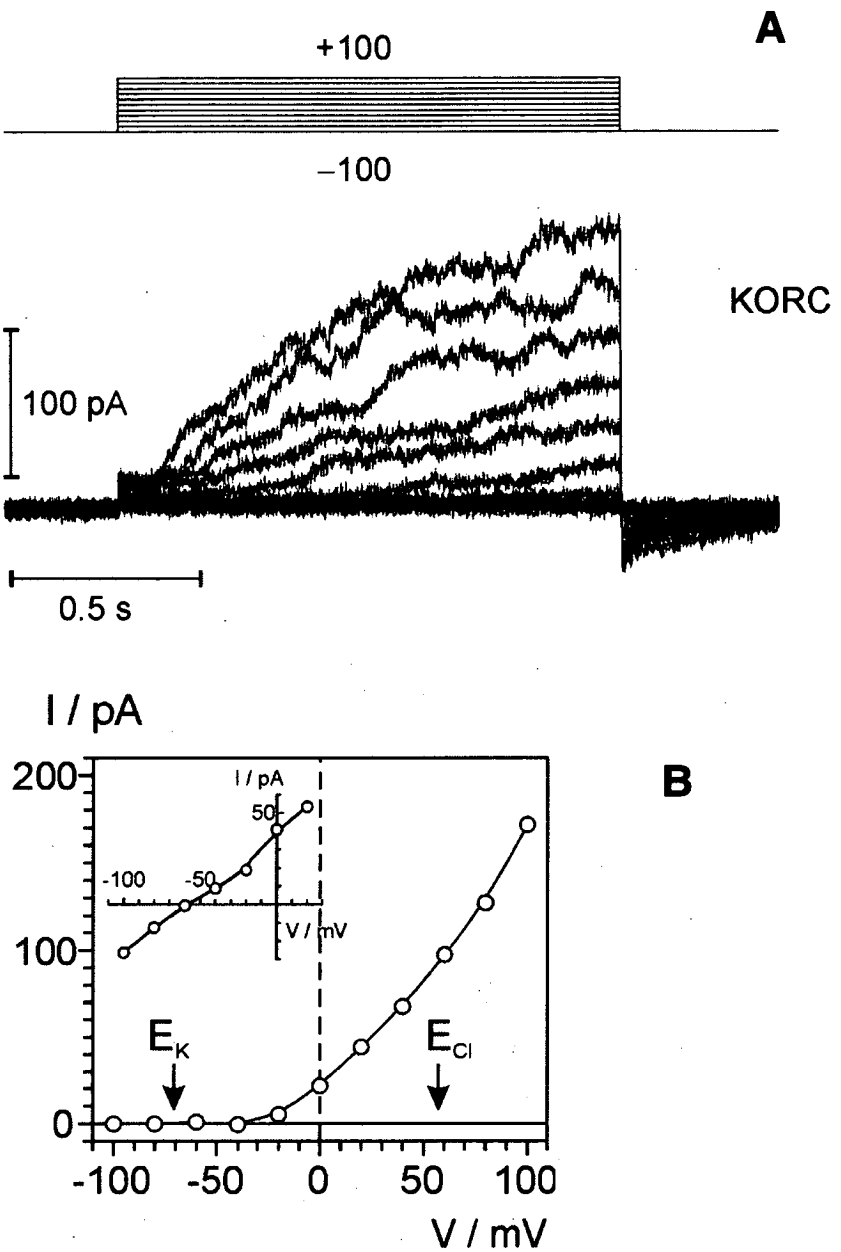

Figure 7.8. (A) – typical records of KOR channels in protoplasts of salt-sensitive cultivar Gairdner. K^+ concentration in bath/pipette was 5/100 mM (see Materials and Methods for detailed solution composition). Voltage was stepped from -100 mV (holding) in 20 mV increments up to +100 mV for 1.4 s and returned to -100 mV at the end of episode. (B) is the I/V relation for the time-dependent component of the depolarisation activated current; equilibrium potentials for K^+ and Cl^- are indicated by arrows. Inset shows amplitude of the tail currents (pre-pulse to +80 mV, subsequent test pulses to voltages between -100 and +20 mV) as a function of test voltage. Professor Pottosin I. and Mr Zepeda-Jazo I. at the Centro Universitario de Investigaciones obtained these results for this thesis.

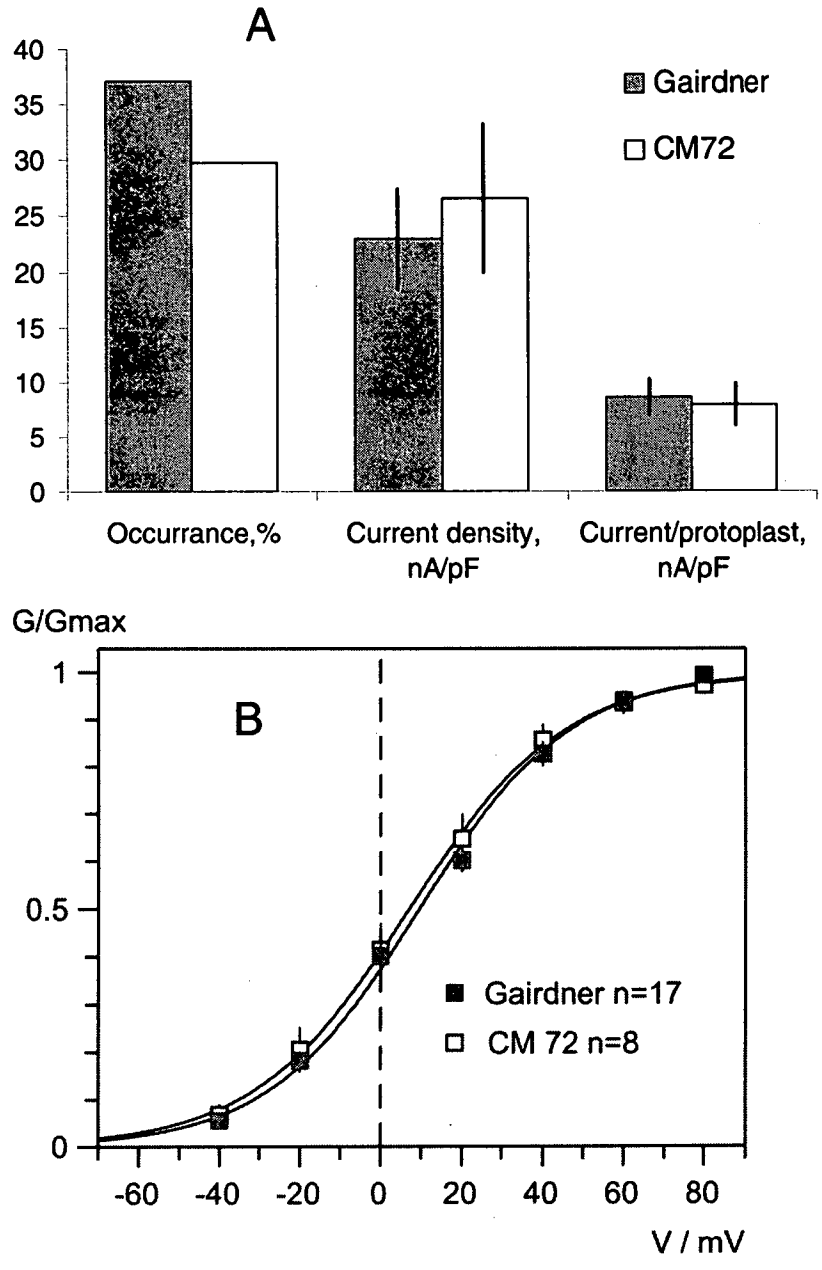

Figure 7.9. KOR channels in two contrasting barley cultivars: salt-tolerant CM72 and salt-sensitive Gairdner. (A) Frequency of detection (successful/total records) of KOR channels, average current densities at +60 mV, and average KOR current per protoplast. Means ± SD (n = 36 for CM72; 70 for Gairdner). (B) Voltage dependence of KOR-mediated conductance. Solid lines are best fits to Boltzmann equation, with midpoint potential values of 6.4 ± 1.4 mV and 9.6 ± 2.4 mV, and the slope factor (membrane depolarisation which increases open/closed states ratio *e*-times) of 18.6 ± 0.8 mV and 18.8 ± 1.2 mV for CM72 and Gairdner, respectively. Professor Pottosin I. and Mr Zepeda-Jazo I. at the Centro Universitario de Investigaciones obtained these results for this thesis.

7.4. Discussion

Most authors agree that K^+/Na^+ homeostasis is a key feature of plant salinity tolerance (Gorham et al. 1990b; Rubio et al. 1995; Dubcovsky et al. 1996; Maathuis and Amtmann 1999; Volkov and Amtmann 2006; Cuin and Shabala 2006). Carden et al. (2003) found that a salt-tolerant barley cultivar was better at maintaining root cytosolic K^+ under saline conditions compared with a salt-sensitive variety. However, most work has only considered whole cell or whole tissue cation concentrations.

Increased salinity tolerance has been reported in transgenic plants expressing yeast *HAL1* gene (Gisbert et al. 2000; Rus et al. 2001; Bordas et al. 1997), and transcription of HAL1 favours Na^+ extrusion and restricts K^+ efflux through an unknown pathway (Bordas et al. 1997), the combination effectively increasing the intracellular K^+/Na^+ ratio (Gaxiola et al. 1992). Overexpression of the yeast homologue of the *HAL3* gene in transgenic *Arabidopsis* plants improves salinity tolerance by increasing the cytoplasmic K^+ concentration and decreasing Na^+ concentrations (Espinosa-Ruiz et al. 1999). *T. halophila*, a salt-tolerant relative of *Arabidopsis*, possesses a greater ability to retain, or even to increase, shoot K^+ concentration compared with *Arabidopsis* under salt stress (Volkov et al. 2004). A strong correlation between a plant's ability to retain K^+ in root epidermal cells and salinity tolerance was reported in Chapter 5 screening nearly 70 barley cultivars covering a wide range of salinity tolerance (Chen et al. 2005, 2007). Thus, targeting mechanistic components responsible for intracellular K^+/Na^+ homeostasis may be an effective way of improving salinity tolerance in crops. What are these components, and what genes encode their function?

7.4.1. Activity of a PM Na^+/H^+ exchanger, but not Na^+ uptake systems, contributes to differential salinity tolerance in barley

Several pathways for Na^+ uptake across the PM have been identified recently using electrophysiological (patch-clamp) and molecular genetic approaches. The major route for Na^+ uptake into the root is believed to be through NSCC, either voltage

independent (so-called VIC channels; Roberts and Tester 1997; White and Davenport 2002), or weakly voltage-dependent (Davenport and Tester 2000; Demidchik et al. 2002a). No significant difference in unidirectional $^{22}Na^+$ influx was found between salt-tolerant and salt-sensitive barley genotypes under the low-Ca conditions in this study (Figure 7.5A and B). Conversely, *net* Na^+ uptake by root (Figure 7.6B) and Na^+ accumulation in leaf (Figure 7.7B) was found to be significantly (by ~ 40 and 35%, respectively) lower in salt-tolerant cultivars (Figure 7.6A). Thus, it appears that salt-tolerant cultivars have a superior ability to pump Na^+ from the cytosol back to the external medium. To date, the only known candidate for such active Na^+ extrusion in higher plants is a PM-bound Na^+/H^+ antiporter (Blumwald et al. 2000), possibly SOS1 (Shabala et al. 2005a). Thus, I suggest that the differential salt-sensitivity between contrasting barley cultivars is in part conferred by higher Na^+/H^+ antiporter activity in salt-tolerant varieties.

Consistent with results reported here, no significant differences in Na^+ influx were found between the wild type and any of the *Arabidopsis sos* and *hkt* mutants with altered salinity tolerance (Essah et al. 2003), or between salt-tolerant and -sensitive wheat cultivars under 0.5 or 2 mM external Ca^{2+} (Davenport et al. 1997, 2005).

7.4.2. Na^+ influx transporters in salt-tolerant genotypes have a higher sensitivity to supplementary Ca^{2+}

Calcium can ameliorate Na^+ toxicity in plants by decreasing Na^+ influx through NSCC (Schachtman and Liu 1999; Davenport and Tester 2000; Demidchik and Maathuis 2007). For instance, a 50% inhibition of NSCC current was observed at 0.1 mM Ca^{2+} activity (Davenport and Tester 2000; Demidchik and Tester 2002). Recently it has been shown that elevated external $[Ca^{2+}]$ also inhibits Na^+-induced K^+ efflux through outwardly-directed K^+-permeable channels (Shabala et al. 2006a). These experiments have demonstrated that two populations of Ca^{2+}-sensitive K^+ efflux channels exist in protoplasts isolated from the mature epidermis of *Arabidopsis* root and leaf mesophyll cells. The instantaneously activating K^+ efflux channels showed weak voltage-dependence and insensitivity to external and internal Na^+. Another population of K^+ efflux channels was slowly activating, steeply rectifying and highly

sensitive to Na^+ (Shabala et al. 2006a).

In the present work, most experiments were conducted at low (0.1 mM) Ca^{2+} to avoid Ca^{2+} inhibition of either NSCC or K^+ efflux channels. Under these conditions, neither immediately upon, nor after 24 h of NaCl treatment, was a clear difference between contrasting cultivars evident in unidirectional $^{22}Na^+$ influx (Figure 7.5A, and B). At high Ca^{2+} levels (10 mM) however, unidirectional $^{22}Na^+$ influx in salt-tolerant genotypes was reduced (Figure 7.5C). During the first 5 min of salt-supply, salt-sensitive genotypes accumulated on average 31% more Na^+ than salt-tolerant ones (Figure 7.5C; $P < 0.05$). This suggests that supplemental external Ca^{2+} is better able to regulate NSCC in salt-tolerant genotypes. This is consistent with Davenport et al. (1997) who showed that in wheat, Na^+ influx into roots was more sensitive to 10 mM Ca^{2+} in salt-tolerant cultivars compared with salt-sensitive ones.

The Ca^{2+} block of NSCC is not complete (even at saturating Ca^{2+} concentrations), with both Ca^{2+}-sensitive and -insensitive components of Na^+ influx being reported for wheat (Davenport and Tester 2000; Demidchik and Tester 2002) and *Arabidopsis* (Essah et al. 2003). Based on the results, I propose that there are two populations of NSCC; Ca^{2+}-sensitive and -insensitive, in barley root epidermal cells. The relative size of these two pools differs between salt-tolerant and -sensitive cultivars, with an apparently larger Ca^{2+}-sensitive pool of NSCC in salt-tolerant genotypes.

7.4.3. Barley salinity tolerance correlates with higher H^+-ATPase activity in root cells

Being an electroneutral exchanger (Serrano and Rodríguez-Navarro 2001), the Na^+/H^+ antiporter cannot be directly responsible for the less pronounced membrane depolarisation found in salt-tolerant barley genotypes under saline conditions (Figure 7.3). This difference in the magnitude of membrane depolarisation is explicable by the intrinsically higher activity of H^+-ATPase in the salt-tolerant cultivars (Figure 7.4A). This higher activity significantly correlated with the smaller ΔE_m and the lower NaCl-induced K^+ efflux (Figure 7.4C). With PM H^+-ATPase being a major determinant of E_m (Michelet and Boutry 1995; Palmgren 2001), more negative E_m values in salt-tolerant genotypes under steady-state conditions could be a direct consequence of a more active H^+ pump. However, in contrast to some other species

(Elkahoui et al. 2005; Yang et al. 2006), Western blot analysis revealed no difference in the amount of protein present between different cultivars (Figure 7.4B). This suggests that the 5-fold difference in H^+-ATPase activity observed between contrasting cultivars (Figure 7.4A) is due to post-translational modulation of the ATPase. The specific nature of such a post-translational modification requires separate investigation.

7.4.4. Different membrane depolarisation but not the difference in KOR properties underlies different K^+ efflux responses in contrasting cultivars

Consistent with the general view that salinity is a polygenic trait (Flowers 2004; Munns 2005), genetic analysis has indicated that NaCl-induced K^+ efflux is under polygenic control - mainly by additive genes with relatively smaller dominant and epistatic effects (Chapter 7). Nonetheless, plant breeders are still in search of a primary gene to improve salinity tolerance in crops. Based on the findings that (1) NaCl-induced K^+ efflux was TEA^+ sensitive and (2) TEA^+ application eliminated the difference in magnitude of K^+ efflux between salt-sensitive and -tolerant genotypes after 1 h of NaCl treatment (Figure 7.2A), it would be logical to propose that contrasting salinity tolerance between the genotypes is determined (at a genetic level), by the different occurrences of PM KOR channels. Surprisingly, patch-clamp experiments on two contrasting genotypes revealed no major difference in KOR channel occurrence. Moreover, salt-tolerant genotypes showed slightly higher KOR-mediated K^+ current density (Figure 7.9A). As a result, at a given membrane potential, the KOR-mediated K^+ current *per protoplast* was not significantly different between the contrasting varieties. In addition, no significant difference in KOR voltage dependence was found in the whole cell mode between salt-sensitive (Gairdner) and salt-tolerant (CM72) cultivars (Figure 7.9B).

Based on the slope of the voltage-dependence, a 20 mV depolarisation difference, as found between the contrasting cultivars (Figure 7.3B), will cause up to a 3-fold difference between their KOR channel open probability. Combining this with the 20 mV difference in driving force for K^+, the difference in K^+ outward current through

KOR channels may indeed equal the difference in the NaCl-induced K^+ efflux observed between Gairdner and CM72 cultivars (Figure 7.1B). Similar conclusions have been drawn by Murthy and Tester (2006) in a patch-clamp study of Na-including and -excluding genotypes of pepper (*Capsicum annuum* L.).

For the quantitative comparison of the K^+ outward current measured by the patch-clamp technique with K^+ loss measured by MIFE one must also take into the account the differences in external [K^+], 5 and 0.5 mM, respectively. A correction can be made based on the biophysical properties of KOR-mediated currents. The properties (selectivity, inhibition by external TEA^+, activation kinetics, voltage dependence) of KOR-mediated currents in epidermal protoplasts are very similar to those of KOR currents described for barley xylem parenchyma (Wegner and Raschke 1994; Wegner and De Boer 1999). Increase of external [K^+] causes a shift of KOR activation threshold and the concomitant shift of the reversal potential so that the entire I/V curve is shifted roughly in parallel to the right by ~ 50 mV per 10-fold increase of external [K^+] (Figure 6 in Wegner and De Boer 1997). Therefore, for CM72 plants, a specific current at -65 mV (the value of free-running membrane potential after NaCl application during MIFE K^+ flux measurements) in 0.5 mM [K^+] bath will be roughly equivalent to the current at -15 mV in a 5 mM K^+ bath (patch-clamp conditions, Figure 7.8). Respective values for Gairdner will be -45 and +5 mV. Now net K^+ fluxes measured by MIFE can be compared with currents measured by patch-clamp. Assuming a specific membrane capacitance of 1 $\mu F\ cm^{-2}$, 120 and 350 nmol $m^{-2}\ s^{-1}$ net K^+ flux (Figure 7.1B) are (if all flux is efflux) equivalent to specific currents of 1.16 and 3.36 pA/pF, for salt-tolerant and -sensitive cultivars respectively. At the same time, from Figure 7.8, the K^+ outward current at -15 and +5 mV will be 0.75 and 2.4 pA/pF, respectively. Therefore, MIFE and patch results are in a good agreement. Two conclusions can be made from these observations: (1) the major portion of NaCl-induced K^+ efflux is mediated by KOR channels, although contributions from other K^+ permeable channels cannot be excluded, and (2) the difference in NaCl-induced K^+ efflux between salt-sensitive and salt-tolerant barley cultivars mainly reflects the difference in NaCl-induced membrane depolarisation, which may, in turn, be primarily determined by the activity of PM H^+-transporting ATPases.

7.4.5. Physiological implications and prospects for breeding

Plant salinity tolerance is a polygenic trait with contributions from genetic, developmental, and physiological interactions, in addition to interactions between the plant and its environment. In this study, multiple mechanisms are shown to be well combined in salt-tolerant barley genotypes enabling them to withstand saline conditions. In addition to efficient Na^+ extrusion (most likely, through a PM Na^+/H^+ exchanger), better retention of K^+ makes a crucial contribution to salinity tolerance in barley. K^+ retention is achieved primarily through the 5-fold higher PM H^+-ATPase activity in salt-tolerant genotypes, leading to smaller membrane depolarisation and, consequently, less K^+ efflux through PM K^+-permeable channels (primarily KORCs). Taken together, these lead both to a superior K^+ retention in the cell and to a reduced concentration of Na^+ in the cytosol. This enables optimal cytosolic K^+/Na^+ homeostasis, hence, normal cell metabolism even under saline conditions.

Suitable manipulation of the PM Na^+ and K^+ transporters, to decrease K^+ loss via KORCs, to enhance H^+-pump-fuelled Na^+ extrusion, or to increase efficiency of inhibition of Na^+-sensitive non-selective channels by external Ca^{2+}, could all contribute to improving salinity tolerance in barley, and other crops as well. These characters could be introgressed into commercial varieties by marker-assisted selection or by using transgenic methods.

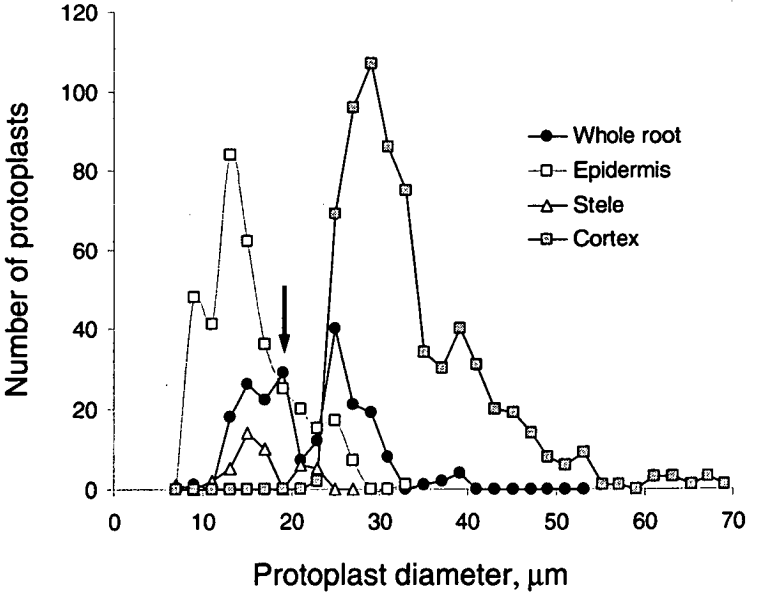

Figure 7.10. Protoplast size distributions for the whole root and the three tissue types isolated. Arrow shows the protoplast size chosen for patch-clamp experiments.

Chapter 8. Compatible Solute Accumulation and Stress Mitigating Effects in Barley Genotypes Contrasting in Their Salt Tolerance

8.1. Introduction

Metabolic acclimation via the accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Hanson and Hitz 1982; Bohnert and Jensen 1996; Sakamoto and Murata 2000; Shabala and Cuin 2006). Many plant species accumulate significant amounts of glycine betaine, proline and polyols in response to high salinity (Rhodes and Hanson 1993; Bohnert et al. 1995; Di Martino et al. 2003). Multiple functions for these compounds have been suggested. In addition to the conventional role of these compatible solutes in cell osmotic adjustment (Yancey et al. 1982; Bray 1993), they were also suggested to act as low-molecular-weight chaperones, stabilising the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Robinson and Jones 1986; Smirnoff and Cumbes 1989; McCue and Hanson 1990; Santoro et al. 1992; Bohnert et al. 1995; Papageorgiou and Murata 1995; Shen et al. 1997; Hare et al. 1998; Mansour 1998; Noiraud et al. 2001). Recently, it was also shown that some of these compatible solutes are very efficient in reducing the extent of K^+ loss in response to both salinity (Cuin and Shabala 2005, 2007a) and oxidative stress (Cuin and Shabala 2007b) in barley and *Arabidopsis* roots.

Different varieties of a particular plant species exhibit a high degree of variation in salt tolerance (Epstein et al. 1980; Chen et al. 2007 and as reported in Chapter 5) and there are various reports on the differential accumulation of glycine betaine and proline among genotypes of cereals (Wyn Jones and Storey 1978; Rhodes et al. 1989; Colmer et al. 1995; Yang et al. 2003), indicating a possible causal link between these processes. Indeed, the introduction of genes involved in the synthesis of proline, betaines, and polyols into plants contributes to abiotic stress tolerance (Rathinasabapathi 2000; Chen and Murata 2002) and numerous genetic engineering attempts have been made to manipulate the biosynthesis pathway of compatible

solutes in order to enhance salt tolerance (Rathinasabapathi 2000; Sakamoto and Murata 2000; Chen and Murata 2002).

However, the levels of compatible solutes accumulated in transgenic plants are not high enough to be osmotically significant (Hare et al. 1998; Bohnert and Shen 1999; Sakamoto and Murata 2000). Thus, exogenous application of compatible solutes has been suggested as an alternative approach to improve crop productivity under saline conditions (Mäkelä et al. 1999; Chen and Murata 2002). External application of low exogenous concentrations of glycine betaine and proline maintained higher K^+ concentration in salt-stressed tomato leaves (Heuer 2003) and decreased salt-induced K^+ efflux from barley roots (Cuin and Shabala 2005, 2007a). Although some researchers have reported positive correlations between the capacity for glycine betaine and/or proline accumulation and salinity tolerance (Binzel et al. 1987; Hare and Cress 1997; Almansouri et al. 1999; Meloni et al. 2001), others have challenged the value of these solutes as positive indicators for resistance to salt stress (Delauney and Verma 1993; Heuer 2003). Thus, controversies exist as to whether hyperaccumulation of glycine betaine and proline is essential for improving salinity tolerance, or whether it is just a symptom of salt stress. These issues are explored in more detail in this study.

As discussed in the previous chapters, one of the hallmarks of salt stress is a massive K^+ efflux from plant roots (Shabala et al. 2003, 2005), affecting cytosolic K^+ homeostasis (Cuin et al. 2003; Shabala et al. 2006a), thus growth and survival of the plant. In previous chapters, a strong correlation has been observed between NaCl-induced K^+ efflux and barley salt tolerance, based on variety of physiological and agronomical indices (Chen et al. 2005, 2007). This led to the proposition of using K^+ retention as an indicator for barley salt tolerance. Given previous findings that applied compatible solutes are generally efficient in reducing the extent of K^+ loss in response to both salinity (Cuin and Shabala 2005, 2007a) and oxidative stress (Cuin and Shabala 2007b), and the fact that ROS production is an established component of salt stress signalling (Hasegawa et al. 2000; Zhu 2001a), the possible causal link between the ability of barley to accumulate/synthesise compatible solutes and salinity stress tolerance warrant a thorough investigation. This was the main aim of this study.

8.2. Materials and methods

8.2.1. Plant materials and growth conditions

Four barley cultivars: salt-tolerant Numar and ZUG293, and salt-sensitive Gairdner and ZUG403, were employed in this study. Conditions for K^+ flux experiments and the glasshouse trial are described in Chapter 3. The average glasshouse temperature and humidity over the growth season were 23°C and 57%, respectively. A randomised complete block design was used, with ten replicates for each cultivar/treatment. Salt treatment was applied at 320 mM NaCl, added gradually with a daily increment of 40 mM NaCl, commencing three weeks after sowing. After four weeks of salt treatment, flag leaf and root samples were collected for high performance liquid chromatography (HPLC) and osmolality measurements.

8.2.2. K^+ flux measurements

K^+ flux was recorded after 1 h salt treatment, then an appropriate amount of either proline or glycine betaine was added, and K^+ flux was recorded for a further 15 min. For the H_2O_2 treatments, K^+ flux was measured in the standard bath solution (0.5 mM KCl and 0.1 mM $CaCl_2$) for 10 min followed by another 30 min after addition of either 1 or 10 mM H_2O_2.

8.2.3. Determination of compatible solutes

8.2.3.1. HPLC instrumentation

The HPLC system consisting of a 717Plus autosampler, 600E pump, 996 photodiode array (PDA) detector and Millennium Chromatography Manager Software (version 32) (Waters Australia Pty Ltd. Rydalmere, NSW, Australia) was used to quantify levels of compatible solutes in plants. The absorption spectrum of eluted compounds was scanned every second from 190 to 400 nm at intervals of 1.2 nm. Microsorb-MV Amino column (250 mm × 4.6 mm) and 4.6-mm MetaGuard column were employed (Varian Inc, USA) with the stationary phases at microsorb-MV 100 NH_2 and Polaris

NH_2 with particle sizes of 5 μm. The mobile phase with acetonitrile:water in the ratio of 84:16 was filtered through 0.45 μm nylon filter under vacuum with a flow rate at 1.50 ml min^{-1}. The columns were maintained at 30°C during chromatography.

8.2.3.2. Sample extraction and purification

Leaf and root samples were freeze-dried and stored below -15°C until analysis. Samples were extracted as described by Naidu (1998). Leaf and root samples were weighed and placed into 10 ml centrifuge tubes. To each tube, 5 ml of methanol:chloroform:water (60:25:15) was added. Tubes were then sealed and heated at 60°C in a water bath for 2 h. Tubes were then removed and 5 ml of deionised water added. The samples were shaken vigorously for 1 min before centrifugation for 10 min at 4000 rpm. The clear upper layer was purified through strong anion exchange resin beads, then filtered through a 0.22 μm Millex-GS syringe driven filter unit prior to being injected into the HPLC.

8.2.3.3. Glycine betaine, sugars and polyols

Glycine betaine, sugars, and polyols were determined as described by Naidu (1998). A mixture of standards: glycine betaine, sucrose, glucose, fructose, mannitol, pinitol and sorbitol, was prepared in methanol:water (50:50) at 0.5 μg μl^{-1} for glycine betaine and 2.5 μg μl^{-1} for the remaining solutes. Ten microlitres of the standard solution was injected into the HPLC while running each batch of samples.

8.2.3.4. Proline

Proline was determined using the rapid method developed by Singh et al. (1973). One ml of sample, 4 ml of ninhydrin solution (Each ml of the ninhydrin solution consisted of 25 mg of ninhydrin, 0.6 ml glacial acetic acid and 0.4 ml 6 M orthophosphoric acid, and heated to 70°C until ninhydrin was completely dissolved) and 4 ml of glacial acetic acid were added to 10 ml centrifuge tubes with 1 ml of deionised water. The thoroughly mixed contents of the tube was kept in a 90 °C water bath for 45 min, then cooled to room temperature. The absorbance was measured at 520 nm using a GBC UV/VIS 916 spectrophotometer (GBC Scientific Equipment Pty Ltd., Dandenong, Victoria, Australia).

8.2.3.5. Total soluble amino acids

One ml of 0.1 M sodium acetate acetic acid buffer (pH = 4.3) and 1 ml of ninhydrin reagent (5% ninhydrin in ethanol) was added to 1 ml of the sample supernatant. The samples were vortexed then immersed in a hot water bath (95°C) for 15 min, and finally cooled to room temperature. Samples were measured at 570 nm using a GBC UV/VIS 916 spectrophotometer.

8.2.4. Estimates on the relative contribution of cytoplasmic solutes to osmotic potential

The relative contribution of the measured solutes to the cytoplasmic osmolality under 320 mM NaCl was made on the following assumptions: (1) cytoplasm comprises 20% of the cell volume (Winter et al. 1993; James et al. 2006 and references within); 95% of Na^+ and Cl^- are sequestered in cell vacuoles (Speer and Kaiser, 1991; Di Martino et al. 2003); (3) leaf Cl^- was about 1.2-fold of Na^+ (Fricke et al. 1996; James et al. 2006); (4) the osmotic pressure was balanced across the tonoplast, preventing NaCl from leaking back to the cytosol; and (5) most compatible solutes and K^+ were preferentially accumulated in the cytosol rather than the vacuole, under severe saline conditions. The relative contribution of each component was calculated according to its absolute amount in the leaves of salt-tolerant and -sensitive cultivars as elsewhere (Meloni et al. 2001; De Lacerda et al. 2003; James et al. 2006).

8.3. Results

8.3.1. Plant growth and nutritional response to salinity

Similar to previous results, four weeks of severe salt stress had a strong impact on plant growth, with height, fresh and dry mass all being significantly reduced ($P < 0.05$; Table 8.1). The effect of salinity, however, differed significantly between barley cultivars, with much better performance of salt-tolerant varieties Numar and ZUG293 after 4 weeks of 320 mM NaCl treatment (Table 8.1). This difference in growth rate was also reflected in a substantial difference of leaf Na^+ and K^+ concentration (Figure

8.3A and B), where salt-sensitive varieties Gairdner and ZUG403 accumulated significantly higher Na^+ and showed greater K^+ loss compared with salt-tolerant ones ($P < 0.05$). Leaf sap osmolality did not differ significantly between genotypes under control conditions (Figure 8.1C), but increased under salinity treatment ~ 2 and 4-fold for salt-tolerant and -sensitive cultivars, respectively (Figure 8.1).

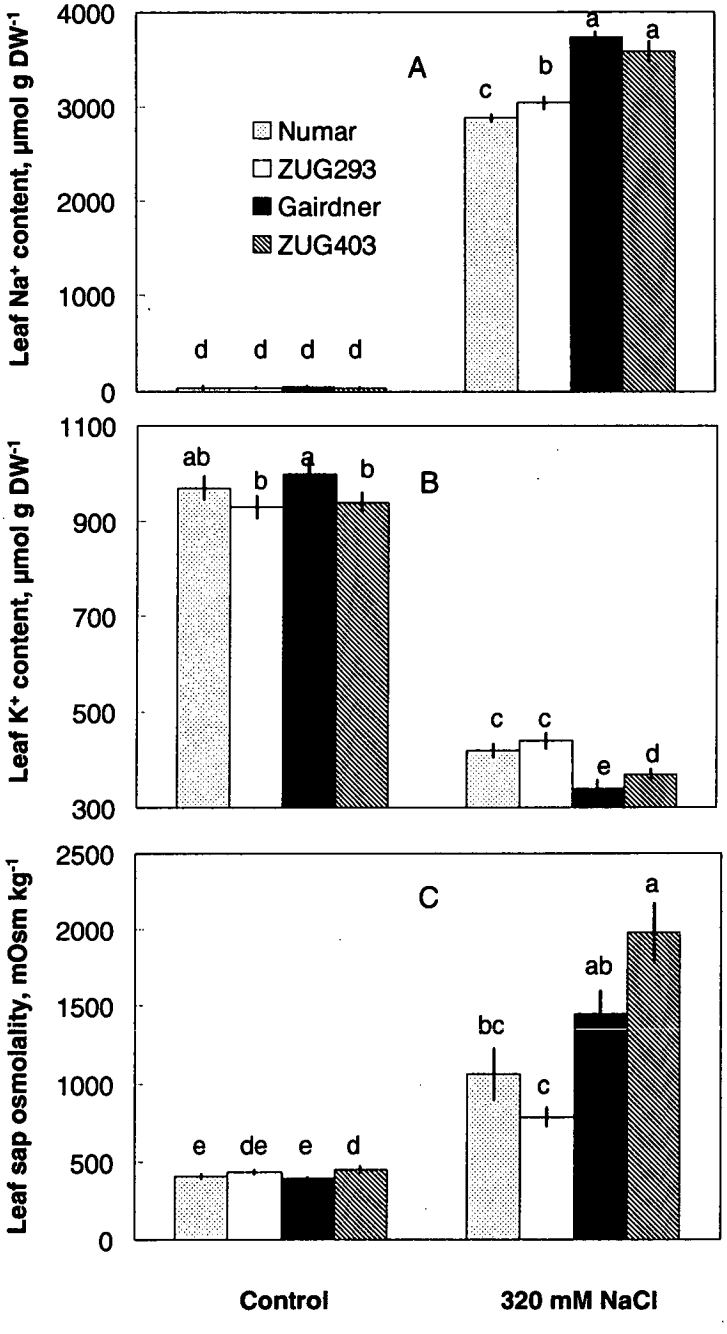

Figure 8.1. Comparison of Na^+ (A), K^+ (B) concentration, and leaf sap osmolality (C) from flag leaves of four barley genotypes in both control and four weeks of 320 mM NaCl treatment. Data are means ± SE (n = 4). Different lowercase letters indicate significance at $P < 0.05$ level.

Table 8.1. Plant height, fresh and dry weight in control and 320 mM NaCl treatment of four barley cultivars differing in salt tolerance. (n = 40 for plant height, n = 24 for fresh and dry weight). Different *lowercase letters* in each column indicate significance at $P < 0.05$ level.

Cultivar	*Plant height, cm*		*Fresh mass, g plant^{-1}*		*Dry mass, g plant^{-1}*	
	Control	320 mM NaCl	Control	320 mM NaCl	Control	320 mM NaCl
Numar	55.0±1.3a	31.5±0.5a	25.3±1.4ab	4.4±0.2a	3.8±0.2a	0.9±0.1a
ZUG293	53.4±1.1a	32.0±0.9a	23.3±1.4ab	4.5±0.2a	3.5±0.2ab	0.9±0.1a
Gairdner	54.7±0.7a	18.1±0.6c	25.5±1.1a	2.1±0.2c	3.1±0.1b	0.4±0.1c
ZUG403	56.6±1.3a	24.0±0.6b	22.7±1.1b	2.4±0.2b	3.6±0.2ab	0.6±0.1b

8.3.2. K^+ flux of salt-tolerant and -sensitive genotypes respond differently to ROS

Exogenous application of ROS (H_2O_2) induced a significant K^+ efflux from epidermal cells in the mature region of barley roots (Figure 8.2). This H_2O_2-induced K^+ efflux was not instantaneous, as has been found for the acute NaCl treatment (Shabala et al. 2003), but rather, it developed gradually reaching peak values after 5 to 10 min, with the peak K^+ efflux showing some dose-dependency on the amount of H_2O_2 applied (Figure 8.2A and B). Potassium flux gradually recovered after reaching its peak, although it always remained as a net efflux. A similar pattern of a slowly increasing ROS-induced K^+ efflux was also observed from *Arabidopsis* roots by Cuin and Shabala (2007b) after the application of a OH•-generating copper/ascorbate mix. Regardless of H_2O_2 concentration used, salt-sensitive genotypes lost on average ~ 2.5 fold more K^+ during the first 20 min of oxidative stress (Figure 8.2A and B).

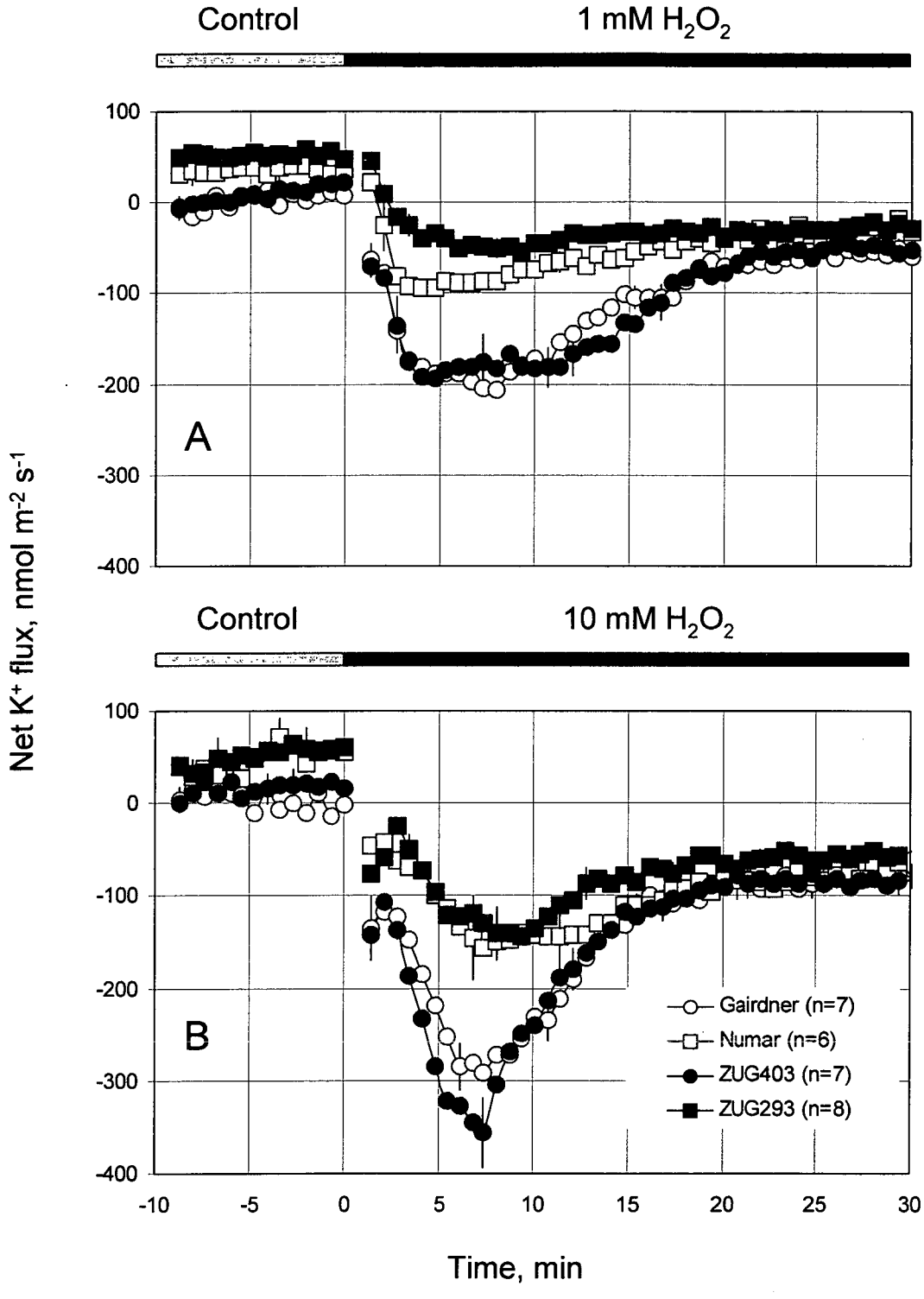

Figure 8.2. Transient root K^+ flux responds to a sudden shock of 1 (A) or 10 (B) mM H_2O_2 applied to four barley cultivars contrasting in their salinity tolerance. Data points are averaged at 30 s of K^+ flux recording. Error bars are SE (n = 6–8).

.8.3.3. Mitigating effects of glycine betaine and proline on NaCl-induced K^+ efflux

Consistent with previous work in our laboratory (Cuin and Shabala 2005), exogenous application of glycine betaine or proline significantly reduced the extent of NaCl-induced K^+ efflux (Figure 8.3A and B), but only in salt-sensitive barley genotypes (31 ± 1.8 and 43 ± 4.6 % reduction after 1 h pre-treatment for 1 and 10 mM

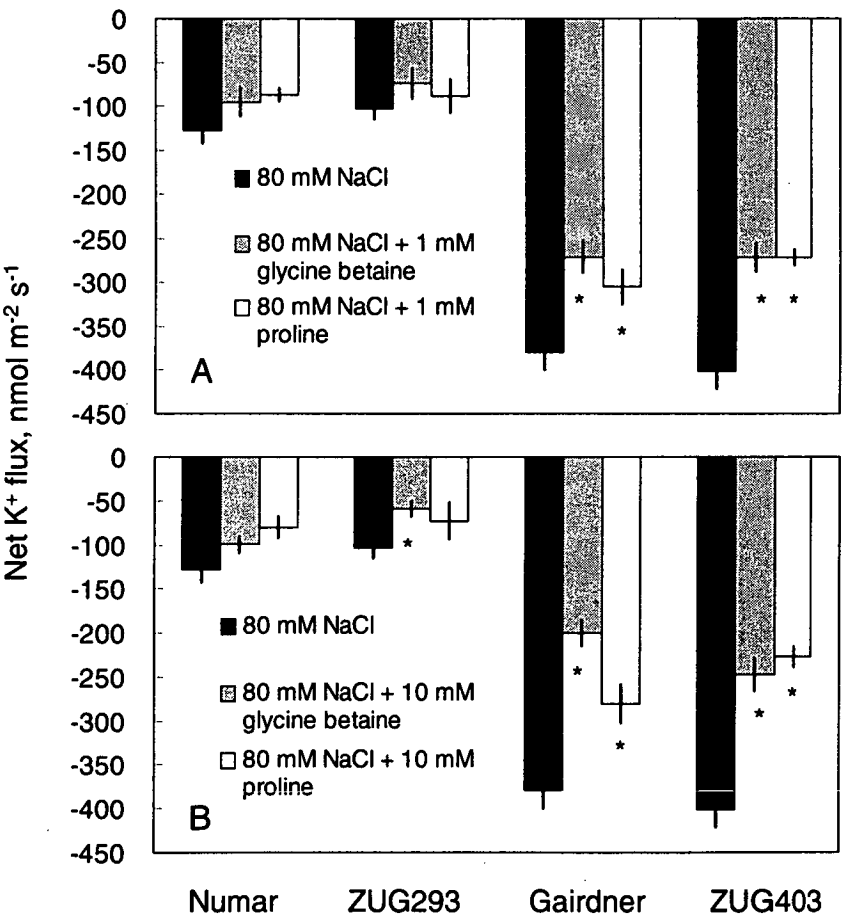

Figure 8.3. Effects of 80 mM NaCl and exogenously applied 1 (A) or 10 (B) mM glycine betaine and proline in addition to 80 mM NaCl on root K^+ flux of barley cultivars differing in salt tolerance. All plants were pre-treated for 60 min with their respective treatments. Data are averaged over a 15 min K^+ flux recording. Error bars are SE (n = 6–10). Statistical significance ($P < 0.05$) of K^+ fluxes within each cultivar is indicated by asterisks.

of exogenous glycine betaine, and 26 ± 6.2 and 35 ± 8.5 % for 1 and 10 mM of exogenous proline, respectively; Figure 8.3). However, effect of these treatments on K^+ loss from salt-tolerant cultivars was only marginal (Figure 8.3A and B).

8.3.4. Polyol accumulation under saline condition

Sorbitol, mannitol and pinitol were detected in both leaf and root tissues using HPLC technique. The concentration of each of these components was on average several folds higher in roots compared with leaves, regardless of the treatment (Table 8.2). No clear difference between contrasting varieties was observed. Four weeks of 320 mM NaCl treatment reduced root polyol concentration in all genotypes except ZUG403. The average reduction for the remaining three cultivars was 30 ± 5.2, 37 ± 6.5 and 44 ± 7.4% for sorbitol, mannitol, and pinitol, respectively. At the same time, sorbitol and pinitol concentration in the leaves increased by 33 ± 9.2 and 86 ± 18%, respectively, while mannitol levels was essentially unchanged (Table 8.2).

8.3.5. Effects of salinity on the total amino acids pool

The total amino acids pool was found to increase in leaves while decreasing in roots after severe salinity treatment (Figure 8.4). The two salt-sensitive Gairdner and ZUG403 showed, on average, a 1.8-fold increase in leaf total amino acid concentration compared with a slight increment for salt-tolerant Numar, while leaf total amino acid concentration of the most salt-tolerant ZUG293 remained unchanged (Figure 8.4A). The effect of salt stress on root total amino acid concentration was much smaller, with the only significant ($P < 0.05$) decline found for the salt-sensitive cultivar Gairdner (29% reduction; Figure 8.4B).

Table 8.2. Comparison of leaf and root polyol (sorbitol, mannitol, and pinitol) concentration of four barley cultivars in both control and 320 mM NaCl treatment. Data are means ± SE. n = 4 for each cultivar and treatment.

	Cultivar	*Sorbitol, μmol g DW^{-1}*		*Mannitol, μmol g DW^{-1}*		*Pinitol, μmol g DW^{-1}*		*Total polyols, μmol g DW^{-1}*	
		Control	Salinity	Control	Salinity	Control	Salinity	Control	Salinity
Leaf	Numar	18.9±0.5	30.5±1.9	10.2±0.4	7.6±0.6	6.0±0.9	8.3±0.9	35.1	46.4
	ZUG293	27.3±1.1	35.5±1.4	9.8±1.8	9.5±1.1	7.3±0.5	12.9±1.1	44.4	57.9
	Gairdner	30.4±3.1	32.5±3.1	9.7±1.2	12.5±1.1	7.1±0.8	17.2±4.3	47.2	62.2
	ZUG403	30.8±2.5	30.6±4.8	15.4±0.7	13.3±1.8	8.0±0.7	n.d.	54.1	43.9
	Mean	26.8	32.3	11.3	10.7	7.1	12.8	45.2	52.6
Root	Numar	80.2±4.5	48.7±9.7	37.9±3.1	22.8±1.1	28.6±3.6	15.0±0.5	146.7	86.5
	ZUG293	57.4±5.4	40.4±3.4	30.2±2.3	16.1±2.5	29.2±1.9	20.7±2.1	116.7	77.2
	Gairdner	67.9±7.4	53.5±5.3	28.9±0.4	21.8±4.3	35.2±3.1	16.3±2.2	132.0	91.6
	ZUG403	38.0±2.1	85.1±6.0	21.9±2.8	33.4±4.0	20.3±2.5	18.5±1.9	80.2	137.1
	Mean	60.9	56.9	29.7	23.5	28.3	17.6	118.9	98.1

Note: n.d. not detected

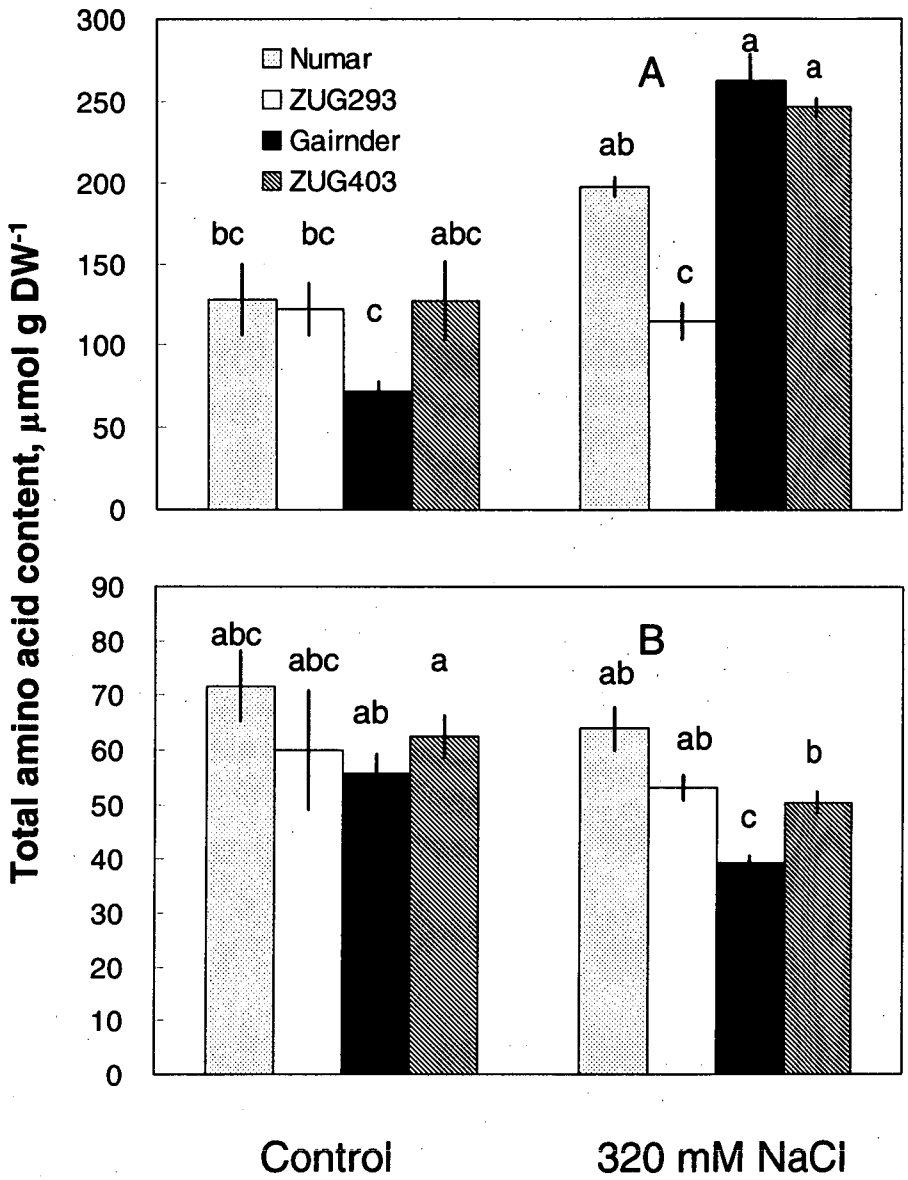

Figure 8.4. Effects of 320 mM NaCl treatment on leaf (A) and root (B) total amino acid concentration among four barley genotypes differing in salt tolerance. Data are mean ± SE (n = 4). Different lowercase letters indicate significance at $P < 0.05$ level.

8.3.6. Effects of salinity on glycine betaine and proline accumulation

Four weeks of 320 mM salinity stress significantly increased leaf glycine betaine and proline accumulation in all varieties, but the effect of salinity differed substantially between genotypes (Figure 8.5A and B). Salt-sensitive cultivars, on average, accumulated over twice as much leaf glycine betaine and proline than salt-tolerant plants under 320 mM NaCl ($P < 0.05$; Figure 8.5A and B). Leaf glycine betaine and proline accumulation correlated negatively ($r = -0.89$ and -0.94, respectively; $P < 0.05$; Table 4) with the ability of roots to retain K^+ under saline conditions (a measure of salt tolerance; Chapter 4, Chen et al. 2005).

Root glycine betaine was undetectable in both treatments, most likely due to its accumulation primarily in chloroplasts (Robinson and Jones, 1986; Ahmad et al. 1987; Nuccio et al. 1999). Root proline concentration in salt-tolerant varieties was twice as high as that of salt-sensitive barley (Figure 8.5C). In general, root proline concentration was substantially lower than in leaves (5 and 20-fold difference for salt-tolerant and -sensitive genotypes, respectively; Figure 8.5).

8.3.7. Correlation analysis

As one of the early indicators of salt tolerance (Chen et al. 2005), NaCl-induced K^+ efflux strongly correlated ($P < 0.01$) with H_2O_2-induced K^+ flux, root proline concentration, relative fresh and dry mass. Significantly correlations ($P < 0.05$) were also found between NaCl-induced K^+ efflux and leaf glycine betaine and proline concentration, relative plant height, and leaf sap osmolality (Table 8.3). The growth components (fresh and dry mass, plant height) and leaf sap osmolality have also been used as indicators of salt tolerance in previous work.

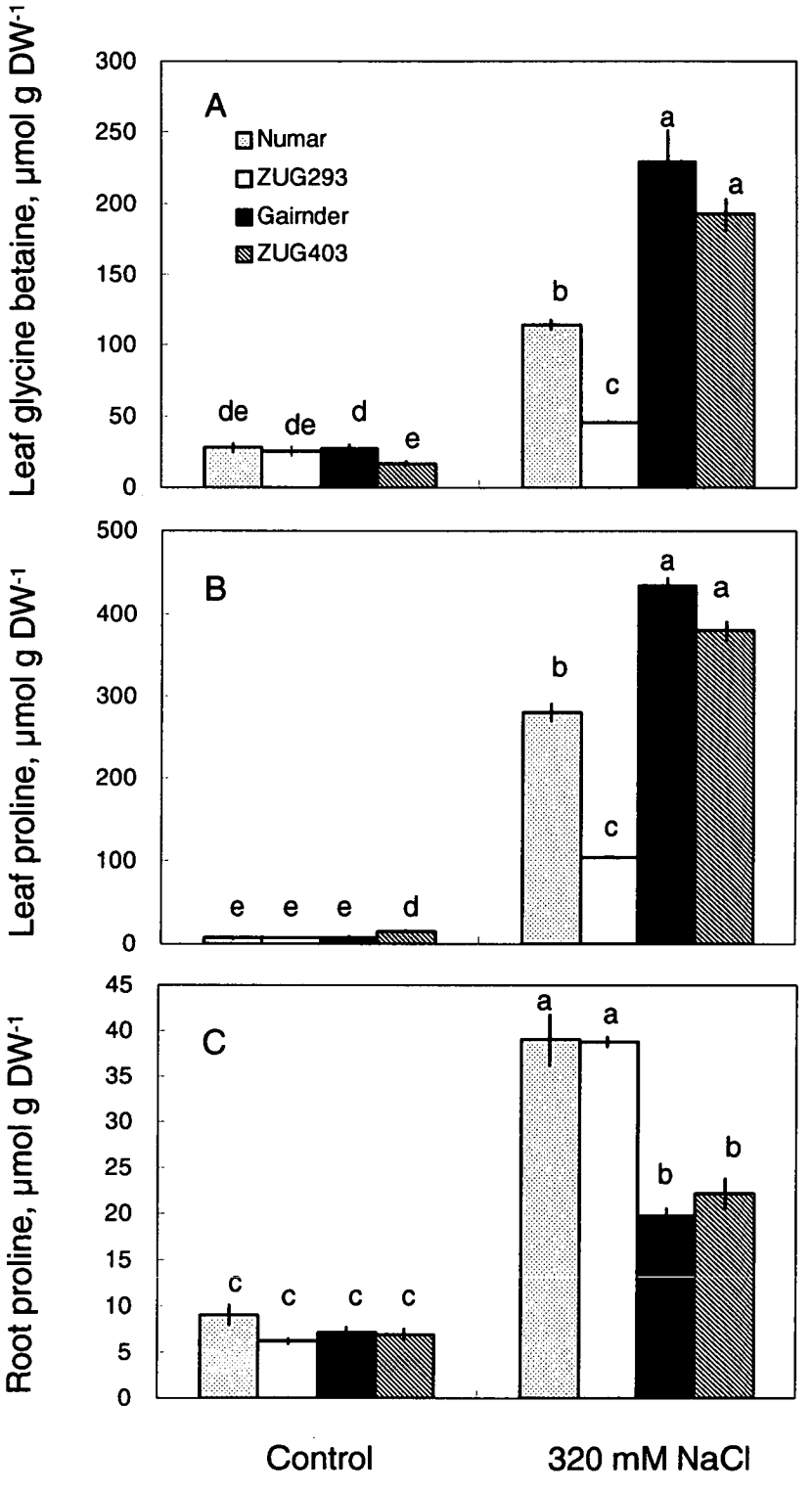

Figure 8.5. Effects of 320 mM NaCl treatment on leaf glycine betaine (A), leaf proline (B), and root proline (C) concentration in four barley genotypes contrasting in salinity tolerance. Glycine betaine and proline concentration in control condition is also shown in each panel. Data are mean ± SE (n = 4). Different lowercase letters indicate significance at $P < 0.05$ level.

Table 8.3. Linear correlation between NaCl-induced K^+ flux (80 mM NaCl) and other parameters determined in this study.

Parameter	*NaCl-induced K^+ flux*
Leaf sap osmolality	0.91*
Relative plant height	0.94*
Relative fresh mass	0.98**
Relative dry mass	0.99**
H_2O_2-induced K^+ flux	0.98**
Leaf glycine betaine concentration	-0.94*
Leaf proline concentration	-0.89*
Root proline concentration	0.99**

Note: significant at * $P < 0.05$, ** $P < 0.01$.

8.4. Discussion

A strong positive correlation between the ability of roots to retain K^+ and salt tolerance in barley has been reported in earlier chapters and in Chen et al. (2005, 2007), highlighting the crucial role of intracellular K^+ homeostasis for plant performance under saline conditions. It was also shown that exogenous application of compatible solutes mitigates both NaCl- and ROS-induced K^+ loss (Cuin and Shabala 2005, 2007a, b). It has been frequently suggested that ROS-scavenging activity is an important component of salt tolerance mechanisms (e.g. Zhu 2001a). It is also well known that ROS may be efficiently scavenged by osmoprotectants, such as proline and mannitol (Xiong et al. 2002; Shabala and Cuin 2006). This poses the question of whether salt-tolerant genotypes have also a superior ability to withstand oxidative stress and (assuming the affirmative answer) to what extent this trait is related to the accumulation of compatible solutes in plant tissues? These issues are addressed in this study.

8.4.1. Salt-tolerant barley show better tolerance to ROS stress

It has been shown in this study that salt-susceptible barley cultivars also had a lower tolerance to ROS (H_2O_2), as shown by the 2 to 3 fold higher K^+ loss from the root epidermis in the mature region (Figure 8.2). Intracellular K^+ homeostasis is critical for plant salt tolerance (Zhu et al. 1998; Maathuis and Amtmann 1999; Carden et al. 2003; Peng et al. 2004; Chen et al. 2005; Shabala et al. 2006a) and may be achieved by different means. ROS-activated K^+ channels have been previously described in many animal systems (Kourie 1998) and ROS-stimulated K^+ efflux has been observed in root cells of various plants (Demidchik et al. 2003, 2007; Shabala et al. 2006a; Cuin and Shabala 2007b). Under saline conditions, the balance between ROS production and scavenging is broken, causing a rapid increase in ROS level (Apostol et al. 1989; Mittler 2002; Apel and Hirt 2004) and concomitant K^+ efflux (Shabala 2006; Cuin and Shabala 2007b; Figure 8.2). The superior ability of salt-tolerant cultivars of preventing ROS-induced K^+ loss from their roots is suggestive of an intrinsically better defence system in these genotypes. For instance, in salt-sensitive potato cultivars, the larger amount of the antioxidant proline produced to compensate for the NaCl-induced oxidative stress, caused an increased H_2O_2 accumulation due to inefficiencies in H_2O_2 scavenging (Fidalgo et al. 2004). This could also partially explain the higher leaf proline levels in salt-sensitive barley. It will be interesting to extend this study to a wider range of genotypes so as to investigate the extent to which this trait reflects the ability of salt-tolerant barley to prevent ROS-induced K^+ loss by maintaining better enzymatic and non-enzymatic defence systems.

8.4.2. Relative contribution of solutes to cytoplasmic osmolality under severe salt stress

The dramatic increase in leaf sap osmolality (Figure 8.1C) in plants subjected to salt stress was largely the result of high accumulation of Na^+ (Figure 8.1) and Cl^- in the leaf cells and salt-induced water loss (Chapter 4, Chen et al. 2005). However, in the cytoplasm, the relative contribution of K^+ to the osmolality was the highest amongst all the solutes studied (Table 8.4). In salt-tolerant varieties, it constituted about half of cytoplasmic osmolality. In salt-sensitive genotypes, however, this figure was

substantially lower (Table 8.4), leading to the requirement for salt-sensitive plants to synthesise at least twice as much cytoplasmic glycine betaine and proline as salt-tolerant ones. The contribution of amino acids (excluding proline) and polyols to osmotic potential were minor in both salt-tolerant and -sensitive genotypes (Table 8.4).

Table 8.4. Relative composition of inorganic and organic solutes in the leaf cytoplasm of salt-tolerant and -sensitive genotypes exposed in 320 mM NaCl for 4 weeks. Data are averaged from two cultivars in each column (see text for more details).

Solutes	*Salt-tolerant lines, %*	*Salt-sensitive lines, %*
Glycine betaine	6.2	13.5
Proline	13.9	24.4
Amino acids (except for proline)	7.2	6.0
Polyols	3.6	3.0
K^+ and its charge balancing anions	49.7	33.1
Na^+, Cl^-, and unknown solutes	19.5	20.0

8.4.3. NaCl-induced K^+ efflux in salt-susceptible cultivars is more sensitive to exogenously applied glycine betaine and proline

Exogenously supplied glycine betaine and proline significantly reduced the magnitude of NaCl-induced K^+ efflux in the two salt-sensitive genotypes (Figure 8.3). However, this mitigating effect was not significant in the salt-tolerant varieties (Figure 8.3). This difference could be due to a differing regulation by exogenous glycine betaine and proline of the various ion channels mediating NaCl-induced K^+ efflux between salt-tolerant and -sensitive genotypes. Increased ROS scavenging is the most obvious candidate. However, both proline and glycine betaine were equally effective in ameliorating ROS-induced K^+ leak from salt-sensitive genotypes (Figure 8.3). At the same time, among the three major types of compatible solutes measured in this study (proline, glycine betaine, and polyols), polyols are reportedly the most effective ROS scavengers, followed by proline, while glycine betaine is thought incapable of scavenging free radicals (Smirnoff and Cumbes 1989; Orthen et al. 1994; Matysik et

al. 2002; Shabala and Cuin 2006). Thus, some other mechanisms such as membrane integrity protection and increasing structural stability of ion transporters may also contribute to this differential regulation. In practical terms, it is prudent to use this high sensitivity of salt-susceptible barley to explore the possibility of supplying exogenous glycine betaine and proline by either foliar sprays or by seeds priming as a means of ameliorating NaCl stress.

8.4.4. Roles of polyols and amino acids in barley salt tolerance

In root tissue, soluble sugars (sucrose, glucose, and fructose) or glycine betaine were below the detection limit (data not shown) of the HPLC. Also, proline accumulation was over 10 times lower than that in leaves. Polyols and amino acids appear to be the major compatible solutes within root tissue (Table 8.2, Figure 8.3 and 8.4). Polyols are mainly synthesised in mature leaves (source tissue) as primary products of photosynthesis and transported to roots (sink tissue) (Noiraud et al. 2001). This is reflected by a root polyol concentration more than twice that of leaves, regardless of salt treatments (Table 8.2). Polyols may also act as ROS scavengers, thus protecting enzyme activities and membrane integrity (Smirnoff and Cumbes 1989; McCue and Hanson 1990; Bohnert et al. 1995; Shen et al. 1997; Noiraud et al. 2001).

The much higher total amino acid concentration increase in leaves of salt-sensitive varieties (Figure 8.4A) may be also indicative of these plants' greater need for ROS scavenging. A higher Na^+ accumulation and a more pronounced K^+ loss in leaves of salt-sensitive genotypes (Figure 8.1A and B) results in reduced photosynthetic efficiency (Chapter 4, Chen et al. 2005), so generating greater oxidative stress in light-exposed leaves. Thus, more amino acids (especially proline) may be needed to mitigate the ROS stress in salt-sensitive cultivars.

8.4.5. Hyperaccumulation of glycine betaine and proline under high salinity does not improve salt tolerance in barley

The importance of K^+ homeostasis in barley salinity tolerance has been investigated in previous chapters (Chen et al. 2005, 2007; also in Cuin and Shabala 2005, 2007a).

The present data is consistent with these reports. Salt-tolerant varieties had a much higher K^+ contribution towards cell osmotic adjustment under saline conditions (50% *vs* 33% for salt-sensitive varieties). As a result, salt-sensitive cultivars needed to synthesise high levels of glycine betaine and proline to compensate for this difference so as to balance the intracellular osmotic potential (Table 8.4). The findings are consistent with reports about higher leaf proline in salt-sensitive genotypes of other species (Colmer et al. 1995; Balibrea et al. 1997; Lutts et al. 1999). It therefore raises the question as to whether the large amount of glycine betaine and proline are actually beneficial for salt adaptation (Rabe 1990; Lutts et al. 1999). Compatible solutes are non-toxic for cytosolic accumulation in plants, but are energetically more expensive. Surviving in saline condition imposes the cost of both excluding salt, and its compartmentation within the cell. However, this cost is relatively small compared to that needed to synthesise organic solutes (Yeo 1983; Raven 1985). It can be calculated that salt-sensitive Gairdner consumed about 4.5-fold of ATP and nitrogen source on synthesising glycine betaine and proline than salt-tolerant ZUG293. This could be the cause of the reduction in growth (Table 8.1) and higher leaf sap osmolality (Figure 8.1C) of salt-sensitive genotypes. Gross measurement of compatible solutes, however, has its disadvantages due to difficulties in its detection within different cell compartments. For instance, glycine betaine is accumulated in chloroplasts to protect leaves from salt stress. Much higher leaf glycine betaine accumulation might also indicate the inefficiency of glycine betaine sequestration (Leigh et al. 1981) in chloroplasts of salt-sensitive genotypes. Specific aspects of such intracellular compartmentation are outside the scope of current study and should be addressed in a separate investigation.

Chapter 9. General Conclusions and Recommendations

Plant salinity tolerance is a polygenic trait, involving multiple mechanisms, with the contribution of genetic, developmental, and physiological interactions within the plant, in addition to interactions between the plant and its environment. The complex nature of plant salinity tolerance and the lack of a full understanding of underlying molecular and cellular mechanisms of salinity tolerance hinder a further improvement in selecting and breeding for salt-tolerant crop species. This study goes towards elucidating the underlying mechanism of salt tolerance in barley. Research into whole-plant, cellular, and molecular response to salinity has shed some light on several aspects of salt tolerance in barley.

There is sufficient variability within the existing barley genotypes to potentially achieve the improvement of salinity tolerance using selection with a number of physiological parameters. It appears that that K^+ loss from the mature zone of intact 3-d old roots following 1 h pre-treatment with 80 mM NaCl can be used as a reliable screening indicator for salt tolerance in barley. A procedure, based on amount of K^+ loss from plant roots exposed to NaCl, was also developed for rapid screening of large numbers of seedlings. Those techniques can work better if combined with glasshouse and field evaluations. It appears that the most efficient approach at this stage would be first to screen all genotypes under laboratory conditions using the MIFE technique, and then conduct glasshouse and field trials on a limited number of prospective genotypes showing good K^+ retention ability under saline conditions. Further research work is needed for the identification of practically useful screening tools based on multiple physiological and agronomic indices. To achieve this, the use of larger numbers of individuals as well as a more diverse pool of germplasm is necessary.

Thus, nearly 70 barley genotypes were employed in large-scale glasshouse experiments in two consecutive seasons to test the above hypothesis by correlating the NaCl-induced K^+ loss and some physiological and agronomic indicators for plant salt tolerance. The results emphasise the importance of maintaining an optimal K^+/Na^+ ratio as a key determinant of barley salt tolerance. It appears that the salt-tolerant barley genotypes should not only possess an ability to efficiently retain K^+ under saline conditions, but also prevent Na^+ accumulation in the shoot. In practical terms,

selection of plants with higher K^+/Na^+ ratio in their tissues may be sufficient to pick up salt-tolerant genotypes. For practical purposes of mass plant screening, the quickest way to estimate this ratio may be by comparing net K^+ and Na^+ fluxes from young barley seedlings. Unfortunately, poor Na^+ LIX selectivity does not allow accurate Na^+ flux measurements at the present. More work is needed to overcome this methodological problem and develop more selective Na^+ sensors.

Also, genetic analysis of barley salt tolerance using NaCl-induced K^+ efflux has revealed that this trait is mainly controlled by additive effects with the presence of smaller contributions from dominant and epistatic effects. The high heritability using NaCl-induced root K^+ flux as a selection criterion suggested that screening salt tolerance based on NaCl-induced K^+ flux could be very effective.

Multiple mechanisms are well combined in salt-tolerant barley genotypes enabling them to withstand saline conditions. In addition to efficient Na^+ extrusion through a PM Na^+/H^+ exchanger, better retention of K^+ contributes crucially to salinity tolerance in barley. K^+ retention is achieved primarily through the 5-fold higher PM H^+-ATPase activity in salt-tolerant genotypes, leading to smaller membrane depolarisation and, consequently, less K^+ efflux through PM KORCs. This enables optimal cytosolic K^+/Na^+ homeostasis under saline conditions. Suitable manipulation of the PM Na^+ and K^+ transporters by decreasing K^+ loss via KORCs, by enhancing H^+-pump-fuelled Na^+ extrusion or by increasing efficiency of inhibition of Na^+-sensitive and non-selective channels by external Ca^{2+}, could all contribute to improving salinity tolerance in barley.

Compatible solutes, which improve plant salt tolerance, are likely to act differently from inorganic ions. It was found that superior K^+ retention and efficient usage of compatible solutes are crucial components for barley salt tolerance. Salt-tolerant cultivars maintained both smaller NaCl- and ROS-induced K^+ efflux than salt-sensitive ones. Micromolar amounts of compatible solutes are sufficient for salt-tolerant cultivars to survive in severe salinity. On the contrary, hyperaccumulation of compatible solutes in salt-sensitive barley consumed 4.5-fold higher ATP and N source without ameliorating the sensitivity to salt but instead appeared to be a symptom of injury.

Several of the above characters (e.g. decreased K^+ loss via KORCs, enhanced H^+-pump-fuelled Na^+ extrusion, increased efficiency of inhibition of Na^+-sensitive and non-selective channels by external Ca^{2+}, and synthesised compatible solutes

efficiently in chloroplasts) can be potentially introgressed into commercial varieties by marker-assisted selection or transgenic methods, which may achieve the development of salt-tolerant crops. However, more comprehensive studies into the molecular, physiological, and genetic mechanisms in crops exposed to salinity stress are necessary to be better equipped for selecting and breeding for salt-tolerant crop genotypes.

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