On the Roles of Membrane Channels in Plant Mineral Nutrition and Toxicity

by

Devrim Coskun

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Cell & Systems Biology
University of Toronto

© Copyright by Devrim Coskun (2016)
Abstract

The study of plant mineral nutrition and toxicity has made major strides recently, particularly at the level of molecular genetics. Arguably, however, this has been at the expense of “classical” physiology, which is of concern because critical physiological examinations of cellular and molecular models in planta are needed if extrapolations of these models to “real-world” (field-level) conditions are to be made. This is particularly urgent in the face of increasing environmental degradation and global food demands. To this end, the present work explores the physiological role of membrane channels in higher-plant nutrition and toxicology, building upon foundational work in nutritional physiology and applying the important new discoveries in the areas of molecular-genetics and electrophysiology. Combining radioisotopic ($^{42}$K$^+$ and $^{13}$NH$_3$/$^{13}$NH$_4^+$) flux kinetics and compartmentation analyses with techniques in electrophysiology, mutant analysis, gas exchange, fluorescence imaging and tracing, and tissue mineral-content analysis, this work investigates the involvement of inward- and outward-rectifying Shaker-like K$^+$ channels (KIR and KOR, respectively), nonselective cation channels (NSCCs), and aquaporins (AQPs), in K$^+$ and NH$_3$/NH$_4^+$ transport under conditions of both stress (low K$^+$, salinity, and ammonium toxicity) and non-stress, in the intact plant. Efflux analyses showed that KOR channels mediate K$^+$ efflux in barley roots only at low external [K$^+$] (<1 mM),
above which transmembrane efflux ceased altogether, and demonstrated their lack of involvement in Na⁺-induced K⁺ efflux in rice roots, an important salinity-stress response. Influx analyses elucidated the involvement of KIR channels in high- and low-affinity K⁺ uptake in barley and Arabidopsis roots, demonstrating dramatic capacity and plasticity, as well as their relevance to salinity stress. By contrast, no evidence of NSCC activity was found under similar conditions. Lastly, rapid transmembrane cycling of NH₃, likely mediated by AQP, was demonstrated to underlie ammonium toxicity in barley roots, fundamentally revising the mechanistic model of low-affinity NH₃/NH₄⁺ transport.
In loving memory of my father,

Müfit Coşkun
Acknowledgments

First and foremost, I would like to express my sincere gratitude to my mentor Prof. Herbert J. Kronzucker. I thank him for opening my eyes to the wonderful world of plant biology, for helping me find the “Ph” (in Ph.D.), and for his continual encouragement, guidance, and commitment throughout my academic development and beyond. His encyclopedic knowledge, scientific brilliance, and humanity, are a constant source of inspiration. I would also like to dedicate this thesis to him on the occasion of his 50th birthday.

Many are not as fortunate as I am to say they have a wonderful teacher, let alone two (!), and so I wish to also thank my (unofficial) supervisor Dr. Dev T. Britto, whom I have the utmost appreciation and respect for. Dr. Britto’s intelligence, wisdom, and humour, are also truly inspirational.

My gratitude further extends to the members of my supervisory committee (past and present), Profs. Malcom Campbell, Andrew Mason, and Greg Vanlerberghe, for their advice and patience throughout committee meetings. The seemingly endless number of radiotracer experiments could not have happened without the teams at the McMaster Nuclear Reactor and CAMH Cyclotron. I also thank the inspirational scientists that I’ve had the honour to meet, learn from, and even collaborate with: Profs. Leon Kochian, Hans Lambers, Norman Hüner, and Tony Glass; our encounters, brief as they may have been, have left a lasting mark.

Many thanks also to my labmates (past and present): Drs. M. Szczerba and L. Schulze, for introducing me to graduate life and teaching me many valuable lessons; W. Huynh, for his kindness and dedication; D. Balkos, A. Becker, A. Hamam, and R. Flam-Shepherd, for the good company; and K. Poon, E. Oh, I. Kabir, K. Jean, M. Li, I. Tolay, A. Torun, S. Gunaseelan, A. Sidiqi, and A. Zaheer, for all the help and collaboration.

I wish to also thank my close friends for the support and good times along the way, Sasha (who encouraged me to join the Kronzucker lab as a lowly undergrad!), Reza, Kevin S. & Maddie, Kyle, Kenway, Kevin H., Calvin & Rosie, and Pat.

Lastly, I thank my family for their continuous love and support throughout this crazy endeavour and beyond. I am especially grateful to my wife, Sahara (whom I met in the Kronzucker lab and share the record with for the most CATE runs in one day!), for her love, encouragement, and
understanding. I am extremely fortunate to share with her all of life’s highs and lows, as well as this passion called “Scienceee” (Esqueleto).
# Table of Contents

Abstract........................................................................................................................................... ii  
Dedication.......................................................................................................................................... iv  
Acknowledgments............................................................................................................................... v  
Table of Contents.............................................................................................................................. vii  
List of Tables ....................................................................................................................................... x  
List of Figures ...................................................................................................................................... xi  
List of Abbreviations .......................................................................................................................... xiv  
Chapter 1 General Introduction ......................................................................................................... 1  
Chapter 2 Materials and Methods .................................................................................................... 8  
  2.1 Plant Growth ............................................................................................................................... 8  
  2.2 Radiotracer Analyses .................................................................................................................. 9  
    2.2.1 Radioisotopes ....................................................................................................................... 9  
    2.2.2 Direct Influx (DI) .................................................................................................................. 10  
    2.2.3 Compartmental Analysis by Tracer Efflux (CATE) ............................................................. 11  
    2.2.4 Pharmacological Treatments ............................................................................................... 12  
  2.3 Electrophysiology ........................................................................................................................ 14  
  2.4 Tissue-Content Analyses .......................................................................................................... 14  
    2.4.1 Flame Photometry ................................................................................................................ 14  
    2.4.2 OPA Assay ........................................................................................................................... 15  
  2.5 Root Respiration ......................................................................................................................... 15  
  2.6 Confocal Microscopy .................................................................................................................. 16  
  2.7 Membrane-Stability Assays ...................................................................................................... 16  
    2.7.1 Electrical Conductivity .......................................................................................................... 16  
    2.7.2 UV-Absorbance Spectrophotometry ..................................................................................... 17  
  2.8 Ag⁺-Induced K⁺-Release Quantification .................................................................................... 17
2.9 Root Water Potential, $\Psi_R$ .............................................................................................................. 17
2.10 PTS Efflux.............................................................................................................................................. 18

Chapter 3 $K^+$ Efflux in Roots of Higher Plants: Mechanisms and Connections to Abiotic Stress ......................................................................................................................................................... 19

3.1 Regulation and Mechanism of Potassium Release from Barley Roots: An in planta $^{43}K$ Analysis ................................................................................................................................................................. 19

3.1.1 Abstract................................................................................................................................................ 19
3.1.2 Introduction......................................................................................................................................... 20
3.1.3 Results................................................................................................................................................ 22
3.1.4 Discussion......................................................................................................................................... 32

3.2 $K^+$ Efflux and Retention in Response to NaCl Stress do not Predict Salt Tolerance in Contrasting Genotypes of Rice (Oryza sativa L.) ............................................................................................................. 38

3.2.1 Abstract................................................................................................................................................ 38
3.2.2 Introduction......................................................................................................................................... 39
3.2.3 Results................................................................................................................................................ 41
3.2.4 Discussion......................................................................................................................................... 58

Chapter 4 $K^+$ Influx in Roots of Higher Plants: Mechanisms and Connections to Abiotic Stress ................................................................................................................................................................. 61

4.1 Capacity and Plasticity of $K^+$ Channels and High-Affinity Transporters in Roots of Barley (Hordeum vulgare L.) and Arabidopsis thaliana L. ..................................................................................................... 61

4.1.1 Abstract................................................................................................................................................ 61
4.1.2 Introduction......................................................................................................................................... 62
4.1.3 Results................................................................................................................................................ 65
4.1.4 Discussion......................................................................................................................................... 86


4.2.1 Abstract................................................................................................................................................ 94
4.2.2 Main text............................................................................................................................................. 95

4.3 How High Do Ion Fluxes Go? A Re-Evaluation of the Two-Mechanism Model of $K^+$ Transport in Plant Roots ........................................................................................................................................ 102
List of Tables

Table 1. Fluxes and compartmentation of K⁺ in roots of barley seedlings grown at either 0.1 or 1.0 mM [K⁺]_{ext}, as determined by compartmental analysis by tracer efflux (CATE).................................. 31

Table 2. Long-term NaCl exposure and tissue biomass and content (low-K⁺, NH₄⁺ conditions). 53

Table 3. Long-term NaCl exposure and tissue biomass and content (low-K⁺, NO₃⁻ conditions). 54

Table 4. Long-term NaCl exposure and tissue biomass and content (high-K⁺, NH₄⁺ conditions). ........................................................................................................................................ 55

Table 5. Long-term NaCl exposure and tissue biomass and content (high-K⁺, NO₃⁻ conditions). 56

Table 6. Short-term NaCl exposure and K⁺ efflux and retention................................................................. 57

Table 7. Steady-state K⁺ fluxes, compartmentation, and electrophysiology of intact barley seedling roots grown on a full nutrient medium supplemented with 10 mM NH₄⁺ and either 0.0225 or 0.1125 mM K⁺ ........................................................................................................................................ 70

Table 8. Electrophysiological responses of barley and Arabidopsis root cells to sudden NH₄⁺ withdrawal with or without Ca(NO₃)₂ ........................................................................................................ 78

Table 9. Compartmental analysis in roots of intact barley seedlings grown and measured with 0.1, 1, or 40 mM K⁺ and the release kinetics of the apoplastic dye PTS ................................................. 110

Table 10. Effects of Ag⁺ on the relative leakage ratio (RLR), and K⁺ and NH₄⁺ contents, of roots from barley seedlings.................................................................................................................. 135
List of Figures

Figure 1. Summary of cellular mechanisms related to K⁺-channel function and regulation in roots of Arabidopsis and its relation to higher levels of organization................................................................. 7

Figure 2. Response of ⁴²K⁺ efflux from roots of intact barley seedlings to sudden provision of channel inhibitors.......................................................................................................................... 24

Figure 3. Response of ⁴²K⁺ efflux from roots of intact barley seedlings to sudden application or elevation of Ca²⁺, K⁺, Rb⁺ or Na⁺ ........................................................................................................... 25

Figure 4. Response of ⁴²K⁺ efflux from roots of intact barley seedlings to sudden provision of NH₄⁺, NH₄NO₃, or NO₃⁻ ....................................................................................................................... 27

Figure 5. K⁺ influx in intact barley seedlings grown at either 0.1 or 1.0 mM [K⁺]ext, and introduced to sudden provision of elevated NO₃⁻, NH₄⁺, or NH₄NO₃ .......................................................................................... 29

Figure 6. Response of ⁴²K⁺ efflux from roots of intact barley seedlings to sudden provision of VO₄³⁻, CN⁻, pH 9.2, or HCO₃⁻ .................................................................................................................. 30

Figure 7. Nutritional and cultivar comparisons of NaCl-stimulated K⁺ efflux from roots of intact rice ............................................................................................................................................. 43

Figure 8. Inhibitor effects of NaCl-stimulated K⁺ efflux from roots of intact rice ................................................................. 46

Figure 9. Root K⁺ content and short-term NaCl stress in rice ........................................................................................................ 48

Figure 10. Correlation analysis between tissue K⁺/Na⁺ content and biomass for rice seedlings in the presence and absence of long-term NaCl stress................................................................................. 50

Figure 11. NaCl-stimulated K⁺ efflux and biomass correlation in rice ......................................................................................... 51

Figure 12. The effects of various pharmacological and nutritional treatments, targeting either Shaker-like K⁺ channels and HAK/KUP/KT transporters, or NSCCs, on steady-state K⁺ influx in intact roots of barley seedlings ........................................................................................................... 69

Figure 13. The effects of various pharmacological and nutritional treatments on steady-state K⁺ influx in intact roots of Arabidopsis ............................................................................................................. 72
Figure 14. Response of $^{42}\text{K}^+$ efflux from roots of intact barley seedlings grown on high $\text{NH}_4^+$ to sudden application of various pharmacological and nutritional treatments ........................................ 74

Figure 15. The effects of various pharmacological and nutritional treatments on $\text{K}^+$ influx stimulated due to $\text{NH}_4^+$ withdrawal in intact roots of barley seedlings .................................................. 77

Figure 16. The effects of various pharmacological and nutritional treatments on $\text{K}^+$ influx stimulated due to $\text{NH}_4^+$ withdrawal in intact roots of Arabidopsis.................................................. 81

Figure 17. Response of $\text{K}^+$ influx to the duration of $\text{NH}_4^+$ withdrawal in roots of intact barley seedlings.......................................................................................................................... 83

Figure 18. Response of tissue $\text{K}^+$ content to the duration of $\text{NH}_4^+$ withdrawal in barley seedlings .......................................................................................................................... 85

Figure 19. Schematic overview of $\text{K}^+$ uptake in plant roots under steady-state conditions (in the presence of high (millimolar) $[\text{NH}_4^+]_{\text{ext}}$)........................................................................................................................................ 93

Figure 20. $\text{K}^+$ influx into intact barley seedlings and the effect of ammonium withdrawal (AWE) $\pm \text{Ca(NO}_3\text{)}_2$ and AWE + $\text{Ca(NO}_3\text{)}_2 \pm$ channel inhibitors ................................................................................................................ 99

Figure 21. Steady-state (control) root $\text{O}_2$ consumption in intact barley seedlings and the effect of sudden $\text{NH}_4^+$ withdrawal (AWE) ........................................................................................................ 101

Figure 22. The two-mechanism model of $\text{K}^+$ acquisition in roots of higher plants and its extension into the saline range .......................................................................................................................... 106

Figure 23. Cellular $\text{K}^+$ efflux ceases in the low-affinity range and what is measured is extracellular (apoplastic) in origin ................................................................................................................ 109

Figure 24. Trans-membrane $\text{K}^+$ influx saturates in the low-affinity range, despite an apparent linear isotherm, and is inversely proportional to plant $\text{K}^+$ status ................................................................................ 113

Figure 25. Plasma-membrane depolarization saturates with rising $[\text{K}^+]_{\text{ext}}$ ................................................................................................................................. 114

Figure 26. The revised model of $\text{K}^+$ acquisition in roots of higher plants ................................................................................................................................. 122
Figure 27. Response of NH$_3$/NH$_4^+$ influx and efflux in roots of intact barley seedlings to 500 µM Ag$^+$. ............................................................ 127

Figure 28. Response of K$^+$ influx into roots of intact barley seedlings to Ag$^+$, Hg$^{2+}$, Au$^{3+}$, Pb$^{2+}$, Cd$^{2+}$, and Ni$^{2+}$ ............................................................. 128

Figure 29. Response of $^{42}$K$^+$ efflux from roots of intact barley seedlings to sudden provision of Ag$^+$, Hg$^{2+}$, Au$^{3+}$, Na$^+$, acetazolamide, zonisamide, forskolin, Pb$^{2+}$, Cd$^{2+}$, and Ni$^{2+}$ ................. 129

Figure 30. Response of $^{42}$K$^+$ efflux from roots of intact barley seedlings to sudden provision of 500 µM Ag$^+$ in combination with channel inhibitors ................................................................. 132

Figure 31. Illustration of the integration technique employed to quantify total K$^+$ released during treatment with 500 µM Ag$^+$. ................................................................................. 136

Figure 32. Confocal micrographs showing propidium iodide staining of the cell wall and nuclei of damaged cells from lateral root tips of intact barley seedlings treated with heavy-metal AQP inhibitors ........................................................................................................... 137

Figure 33. NH$_3$ (not NH$_4^+$) is the main permeating species in barley roots ......................... 148

Figure 34. Effect of 5-min exposure to elevated pH (pH 9.25) on root respiration in barley plants grown with 0.01 mM K$_2$SO$_4$ and 5 mM of either (NH$_4$)$_2$SO$_4$ or Ca(NO$_3$)$_2$ ........................................ 150

Figure 35. Characterization of NH$_3$/NH$_4^+$ efflux from roots of intact barley seedlings .......... 153

Figure 36. Pharmacological profile of NH$_3$/NH$_4^+$ influx into roots of barley seedlings at varying external pH ........................................................................................................ 154

Figure 37. Revised model of futile transmembrane NH$_3$/NH$_4^+$ cycling in root cells of higher plants ................................................................................................................. 160
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP1</td>
<td>AKT1-interacting PP2C 1</td>
</tr>
<tr>
<td>AKT</td>
<td>Arabidopsis K⁺ transporter 1</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BUS</td>
<td>Back-up system (of K⁺ acquisition)</td>
</tr>
<tr>
<td>CATE</td>
<td>Compartmental analysis by tracer efflux</td>
</tr>
<tr>
<td>CBL</td>
<td>Calcineurin-B-like protein</td>
</tr>
<tr>
<td>CCCP</td>
<td>Cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CHX</td>
<td>Cation-H⁺ exchanger</td>
</tr>
<tr>
<td>CIPK</td>
<td>CBL-interacting protein kinase</td>
</tr>
<tr>
<td>CNGC</td>
<td>Cyclic nucleotide gated channel</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>EC</td>
<td>Electrical conductivity</td>
</tr>
<tr>
<td>E:I</td>
<td>Efflux:influx ratio</td>
</tr>
<tr>
<td>E_k⁺</td>
<td>Equilibrium (Nernst) potential for K⁺</td>
</tr>
<tr>
<td>HAK/KUP/KT</td>
<td>High-affinity K⁺/K⁺ uptake/K⁺ transporter</td>
</tr>
<tr>
<td>[K⁺]_{cyt}</td>
<td>Cytosolic K⁺ concentration</td>
</tr>
<tr>
<td>[K⁺]_{ext}</td>
<td>External K⁺ concentration</td>
</tr>
<tr>
<td>KAT</td>
<td>K⁺ channel in <em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>KIR</td>
<td>Inward-rectifying K⁺ channel</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Half-saturation (Michaelis-Menten) constant</td>
</tr>
<tr>
<td>KOR</td>
<td>Outward-rectifying K⁺ channel</td>
</tr>
<tr>
<td>[NH₄⁺]_{ext}</td>
<td>External NH₄⁺ concentration</td>
</tr>
<tr>
<td>NHX</td>
<td>Na⁺-H⁺ exchanger</td>
</tr>
<tr>
<td>NORC</td>
<td>Nonselective outward-rectifying channel</td>
</tr>
<tr>
<td>NSCC</td>
<td>Nonselective cation channel</td>
</tr>
<tr>
<td>OPA</td>
<td>o-phthalaldehyde</td>
</tr>
<tr>
<td>Pₙ</td>
<td>Osmotic water permeability</td>
</tr>
<tr>
<td>pKa</td>
<td>-log₁₀ (Acid dissociation constant)</td>
</tr>
<tr>
<td>PP2C</td>
<td>2C-type protein phosphatase</td>
</tr>
</tbody>
</table>
ROS  Reactive oxygen species
SHAM  Salicylhydroxamic acid
SNARE  Soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor
SOS  Salt-overly sensitive
\( t_{1/2} \)  Half-life
TEA\(^+\)  Tetraethylammonium
\( V_{\text{max}} \)  Maximum velocity
WUE  Water-use efficiency
WT  Wild type
\( \gamma_{\text{cyt}} \)  Cytosolic K\(^+\) activity coefficient
\( \Delta \Psi_m \)  Membrane electrical potential
\( \Delta \Delta \Psi_m \)  Change in membrane electrical potential (depolarization/hyperpolarization)
\( \Psi_R \)  Root water potential

**Molecular-Genetic Nomenclature**

Uppercase  Protein (e.g. AtAKT1)
Uppercase italicized  Gene (e.g. AtAKT1)
Lowercase italicized  Mutant (e.g. atakt1)
Our understanding of plant membrane channels has come a long way since Burdon-Sanderson first corresponded with Darwin about the Venus flytrap (Dionaea muscipula), and measured the first action potentials in plants from its leaf (Burdon-Sanderson 1873; Darwin 1875; see also Pickard 1973; Fromm and Lautner 2007; Hedrich 2012, for a historical overview). The mechanistic basis of this action potential, i.e. plant ion-channel activity, would not be isolated and characterized electrophysiologically until more than a century later, following the invention of the patch-clamp technique by Neher and Sakmann (Moran et al. 1984; Schroeder et al. 1984). In roots, the “hidden half” of the plant, measurement of thermodynamically passive ion fluxes (i.e. down an electrochemical gradient, as in Oertli’s “pump-leak” model, where transport of Rb⁺, a K⁺ analogue, was explained by an active inward “pump” and a passive bi-directional “leak” at the plasma membrane; Oertli 1967; see also Dainty 1962; Epstein et al. 1963; Laties 1969), led to the postulation that ion channels were present in root-cell membranes. However, as with ion channels found in shoots, the electrophysiological characterization of root ion channels (namely K⁺-selective channels) would not occur until the early 1990s (White and Tester 1992; Gassmann and Schroeder 1994). This would coincide with the first cloning of plant ion channels, i.e. KAT1 (potassium channel in Arabidopsis thaliana 1; Anderson et al. 1992), which is found in guard cells, and AKT1 (Arabidopsis K⁺ transporter 1; Sentenac et al. 1992), which is predominately found in roots. Both KAT1 and AKT1 are inward-rectifying K⁺ channels of the Shaker-like (voltage-dependent) family, which is the best characterized transporter class in plants to date (for review, see Véry and Sentenac 2003; Dreyer and Uozumi 2011).

Early physiological studies which kinetically mapped out the primary acquisition of K⁺ from external solutions, using radiotracers (Epstein et al. 1963; Glass 1976; Kochian and Lucas 1982), have been buttressed and expanded since the advent of modern molecular-genetic techniques. For example, it is now well established that H⁺:K⁺ symporters of the HAK/KUP/KT family (e.g. AtHAK5 in Arabidopsis [Arabidopsis thaliana]), alongside AtAKT1, catalyze the vast majority (~80%) of K⁺ acquisition in Arabidopsis roots (Gierth and Mäser 2007), and this model adequately explains, at the molecular level, Emanuel Epstein’s two-mechanism model (Epstein
et al. 1963), which describes K\(^+\) acquisition (in roots of barley \([\textit{Hordeum vulgare}]\)) as the sum of activities of high- and low-affinity transport systems (HATS and LATS, respectively; Hirsch et al. 1998; Gierth and Mäser 2007; Alemán et al. 2011; Coskun et al. 2014b). Moreover, intricate regulatory mechanisms of K\(^+\) transport in Arabidopsis have been described at the molecular level, such as how “low-K\(^+\) stress” can trigger a Ca\(^{2+}\) signaling cascade where cytosolic free Ca\(^{2+}\) binds to calcineurin-B-like protein 1 (CBL1) and CBL9. These proteins, in turn, interact with CBL-interacting protein kinase 23 (CIPK23), forming a protein complex that phosphorylates and activates AtAKT1-channel complexes \(i.e.\) heterotetramer complexes made up of AtAKT1 and AtKC1 subunits in Arabidopsis; Li et al. 2006; Xu et al. 2006; Lee et al. 2007; Geiger et al. 2009; see also Grefen and Blatt 2012; Subchapter 4.1; Fig. 1). In addition, channel deactivation via a 2C-type protein phosphatase (PP2C), AKT1-interacting PP2C 1 (AIP1), has been identified (Lee et al. 2007; Lan et al. 2011). Adding further complexity, AtAKT1-channel complexes have been shown to also be regulated by the soluble \(N\)-ethylmaleimide-sensitive factor protein attachment protein receptor (SNARE), SYP121, which binds directly to AtKC1, forming a tripartite complex with AtAKT1. This complex has been shown to be essential for proper channel functioning (Honsbein et al. 2009). SNAREs are a ubiquitous superfamily of proteins responsible for vesicle targeting and fusion \(i.e.\) membrane trafficking; Grefen and Blatt 2008), and interestingly, it has been hypothesized that SYP121 is an important link between ion transport and membrane/cell expansion (Grefen and Blatt 2008).

Despite the many breakthroughs in our understanding of K\(^+\)-channel function and regulation, many fundamental questions remain regarding the mechanisms of K\(^+\) transport in and out of roots, both under conditions of stress and non-stress. Moreover, it is critical that \textit{in-planta} demonstrations of cellular and molecular models that are based on heterologous-expression and protoplast systems (the bases of the models described above) be forthcoming, if extrapolations to plant performance under “real-world” \(i.e.\) field-level) conditions are to be realized (Fig. 1). This is particularly important in the light of increasing environmental stresses \(e.g.\) drought, salinity, soil erosion, and anthropogenic “climate change” associated with greenhouse gas emissions) which compromise plant performance, as well as a burgeoning human population that is increasing demands on agricultural output and putting strains on land, water, and nutrient resources (Coskun et al. 2014b).
One example indicating the need for increased physiological understanding involves the poorly understood mechanisms and regulation of K\textsuperscript+ release (efflux) from the cell, in stark contrast to the wealth of knowledge surrounding K\textsuperscript+ influx and accumulation (see above). Similarly, the mechanism and relevance of Na\textsuperscript+-induced K\textsuperscript+ release from roots of higher plants under salinity stress remain equivocal (Nassery 1979; Cramer et al. 1985; Shabala et al. 2006; Britto et al. 2010; Coskun et al. 2013a). To what extent outward-rectifying K\textsuperscript+ channels are responsible for this phenomenon remains unclear, as is the extent to which this phenomenon can be used to screen for salt-tolerance across contrasting cultivars of agriculturally important crop species (Chen et al. 2007; Cuin et al. 2008; Coskun et al. 2013a). With respect to K\textsuperscript+ acquisition, questions remain regarding the robustness of the Arabidopsis model (Alemán et al. 2011), and whether it can be extended to crops such as rice, wheat, maize, and barley (Coskun et al. 2013c; Coskun et al. 2014b). For example, can K\textsuperscript+ channels like AKT1 operate at very low (e.g. 10 µM) external K\textsuperscript+ concentrations ([K\textsuperscript+]\textsubscript{ext}) in cereals, as has been demonstrated in Arabidopsis (Hirsch et al. 1998; Spalding et al. 1999)? What are the physiological roles of nonselective cation channels (NSCCs; a class of voltage-sensitive and -insensitive membrane channels which mediate the passive, and relatively nonselective, transport of cations, such as Na\textsuperscript+, K\textsuperscript+, NH\textsubscript{4}\textsuperscript+, Rb\textsuperscript+, Cs\textsuperscript+, Li\textsuperscript+, Ca\textsuperscript{2+} and Mg\textsuperscript{2+}; Demidchik et al. 2002; Demidchik and Maathuis 2007; Kronzucker and Britto 2011) in K\textsuperscript+ acquisition, and can their study provide insight into Na\textsuperscript+ transport in plants (rapid NSCC-mediated influx of Na\textsuperscript+ is a leading explanation of the mechanism of Na\textsuperscript+ uptake in roots under salinity-stress conditions; Munns and Tester 2008; Kronzucker and Britto 2011)? Lastly, do K\textsuperscript+ channels operate at very high [K\textsuperscript+]\textsubscript{ext} (i.e. >50 mM) and can this explain the linear nature of influx isotherms measured in the low-affinity range? A deeper understanding of low-affinity K\textsuperscript+ fluxes may provide important insight into the elusive nature of Na\textsuperscript+ transport under conditions of Na\textsuperscript+ toxicity (i.e. salinity stress).

Another major environmental concern is ammonium toxicity in plants, the result of an accelerated global N cycle, brought about primarily by the industrialized production (via the Haber-Bosch process) and application of N fertilizers, as well as anthropogenic greenhouse-gas emissions (Galloway et al. 2008; Gruber and Galloway 2008; Fowler et al. 2015). A major hypothesis to explain the toxicity syndrome in higher plants is that of futile NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} cycling across the plasma membrane of roots, whereby rapid influxes are met by nearly equal rapid effluxes, which is postulated to result in significant metabolic burdens (Britto et al. 2001b;
Coskun et al. 2013b). Surprisingly, the fundamental question of which member of the conjugate acid/base pair, \( \text{NH}_3 \) or \( \text{NH}_4^+ \), participates in this futile cycling remained unknown until recently (see Coskun et al. 2013b; Chapter 6). Moreover, the precise mechanism mediating this flux remains unknown (ten Hoopen et al., 2010; Esteban et al., 2016); however, it has been hypothesized that a pathway is via aquaporins (AQP; see Chapter 6), which comprise a diverse and ubiquitous group of membrane-bound proteins that facilitate the movement of water, and small, usually uncharged molecules such as urea, glycerol, carbon dioxide, silicic acid, and ammonia (Kozono et al. 2002; Maurel et al. 2008; Hove and Bhave 2011). While there is evidence for AQP-mediated \( \text{NH}_3 \) transport in heterologous expression systems (Jahn et al. 2004; Holm et al. 2005; Loqué et al. 2005; Hove and Bhave 2011), a convincing \textit{in planta} demonstration remains to be seen.

Thus, the overarching theme of this thesis is to investigate the physiological role of membrane channels in higher-plant mineral nutrition and ion toxicity. Cellular and molecular models of channel-mediated transport processes have been tested in intact plants and connections to plant performance under field conditions have been drawn.

Chapter 3 investigates the fundamental mechanisms underlying \( \text{K}^+ \) release (efflux) from roots under stressed and non-stressed conditions. Subchapter 3.1 investigates the specific mechanisms and regulations of \( \text{K}^+ \) efflux in intact barley seedlings under various nutritional regimes and sheds light on the thermodynamic shifts in plant \( \text{K}^+ \) transport as a function of \( [\text{K}^+]_{\text{ext}} \) from the efflux perspective, while also characterizing key N-source (\( \text{NO}_3^- \) vs. \( \text{NH}_4^+ \)) effects. Subchapter 3.2 investigates the mechanism of \( \text{Na}^+ \)-induced \( \text{K}^+ \) efflux in roots of rice seedlings, and addresses the issue of whether the extent of \( \text{Na}^+ \)-induced \( \text{K}^+ \) efflux can be used as a measure of overall plant salt tolerance. Using three cultivars of rice varying in sensitivity to salinity, as well as a nutritional matrix varying in \( \text{K}^+ \) level, N source, and N level, this work investigates \( \text{Na}^+ \)-induced \( \text{K}^+ \) efflux and its correlation with cultivar performance on high NaCl.

Chapter 4 explores the mechanisms of \( \text{K}^+ \) influx in roots of higher plants. Subchapter 4.1 investigates the relative contributions of HAK/KUP/KT high-affinity transporters, AKT1-channel complexes, NSCCs, and hitherto genetically unidentified “back-up systems” (BUS) (as evident in the \textit{athak5 atakt1} double-knock-out mutant in Arabidopsis) to \( \text{K}^+ \) uptake. Subchapter 4.2 further extends this work by exploring anion effects (\( \text{Cl}^- \), \( \text{SO}_4^{2-} \), and \( \text{NO}_3^- \)) on \( \text{K}^+ \) influx, root
respiration, and pharmacological sensitivity, underscoring new additions to the current model of K$^+$ acquisition. Subchapter 4.3 explores the upper limits of K$^+$ acquisition, asking: how high do channel-mediated K$^+$ fluxes go? Above 10 mM [K$^+$]$_{ext}$, linear influx isotherms based on radiotracer analyses suggest ion channels are capable of extraordinarily high flux capacity. We critically re-examine the two-mechanism model of K$^+$ influx (first developed by Epstein; see above), and investigate the involvement of extracellular (apoplastic) fluxes in ion transport, particularly in the saline range.

Chapter 5 serves as an introduction into the study of NH$_3$/NH$_4^+$ fluxes under ammonium toxicity. To test the hypothesis that low-affinity NH$_3$ fluxes are mediated by AQPs, $^{13}$NH$_3$ fluxes were examined under the influence of well-established heavy-metal AQP inhibitors, Hg$^{2+}$, Au$^{3+}$, and Ag$^+$ (Preston et al. 1992; Niemietz and Tyerman 2002). The discovery of the surprising side effects of these inhibitors on K$^+$ transport and cellular membrane integrity serve as a cautionary note on the use of pharmacological inhibitors.

Chapter 6 begins to elucidate the nature of NH$_3$/NH$_4^+$ transport in roots of higher plants under ammonium toxicity. This chapter explores the relative contribution of each species of the conjugate acid/base pair (NH$_3$/NH$_4^+$) to the futile transmembrane cycling observed under these conditions. Taking the lessons learned from Chapter 5, the hypothesis that AQPs permeable to NH$_3$ (i.e. aquaammoniaporins) mediate such fluxes were further investigated. This work paves the way for future studies exploring the mechanism and regulation of rapid NH$_3$ gas fluxes under ammonium toxicity and will contribute to understanding and addressing this important global environmental issue.

**Note regarding organization of thesis:** The bulk of this thesis has been published in peer-reviewed journals. These papers have been reformatted and presented as subchapters in this thesis. For the sake of brevity, and to avoid redundancy, the ‘Materials and Methods’ sections from each paper have been amalgamated into a single chapter (Chapter 2), since many of the preparations and experiments throughout the thesis are variations of the same procedures. The reader may refer to this chapter while reading the research chapters. Similarly, detailed introductions to each topic can be found in the beginning of each research chapter.
Different species (e.g., cereals)

Different environments (e.g., NO$_3^-$ soils)

Intact plant (e.g., low-K$^+$, high-NH$_4^+$ condition)

Single-cell (heterologous/protoplast) system
**Figure 1.** Summary of cellular mechanisms related to K⁺-channel function and regulation in roots of Arabidopsis and its relation to higher levels of organization. In single-cell (heterologous/protoplast) systems, the K⁺-channel heterotetrameric complex AtAKT1-AtKC1 can mediate K⁺ acquisition from low (micromolar) external concentrations ([K⁺]_{ext}) and in the presence of NH₄⁺. External K⁺ deficiency is sensed by the cell and results in ROS production which leads to cellular Ca²⁺ signaling cascades. Ca²⁺ binds to CBL proteins to either directly modulate AtAKT1 (see CBL10) or activate protein kinases (CIPK23) that phosphorylate AtAKT1 and activate the channel complex. By contrast, the protein phosphatase AIP1 can dephosphorylate AtAKT1 and thereby inactivate the complex. CBL3-CIPK9 also regulates K⁺ acquisition, although via indirect mechanisms. The SNARE protein SYP121 interacts with AtKC1 to activate the channel complex and also facilitates membrane trafficking and cell expansion via vesicular fusion. Anions (Cl⁻ and NO₃⁻) may also modulate channel activity, albeit only at high (millimolar) [K⁺]_{ext}. Other potential means of K⁺ acquisition involve non-selective cation channels (NSCCs), which may or may not represent the K⁺ ‘backup system’ (BUS). Also, the high-affinity transporter AtHAK5 mediates H⁺-coupled K⁺ symport under conditions of thermodynamically active K⁺ uptake (powered by the H⁺-ATPase). Lastly, the K⁺ channel GORK mediates cellular K⁺ efflux under passive-release conditions. Importantly, *in-planta* links to many cellular-mechanistic characterizations require further exploration. Once such links are made, broader applications can be developed, such as with species (*e.g.* cereals) and to conditions relevant to agriculture. Figure derived from Coskun et al. (2014b).
Chapter 2
Materials and Methods

2.1 Plant Growth

Barley seeds (*Hordeum vulgare* L. cv ‘Metcalf’) were surface-sterilized for 10 min in 1% sodium hypochlorite, and germinated under acid-washed sand for 3 d before placement in 12- or 14-L plastic hydroponic vessels containing aerated, modified Johnson’s barley solution (0.5 mM NaH$_2$PO$_4$, 0.25 mM MgSO$_4$, 0.2 mM CaSO$_4$, 25 µM H$_3$BO$_3$, 20 µM FeEDTA, 2 µM ZnSO$_4$, 0.5 µM MnSO$_4$, 0.5 µM CuSO$_4$, and 0.125 µM Na$_2$MoO$_4$), pH 6.3–6.5 (adjusted with 1 M NaOH), for an additional 4 d. The growth solutions were modified to provide potassium (as K$_2$SO$_4$, except for Subchapter 4.2, where KCl was also used), at various concentrations (see text). N was supplied as either Ca(NO$_3$)$_2$ or (NH$_4$)$_2$SO$_4$, at various concentrations (see text). To ensure that plants remained at a nutritional steady state, solutions were exchanged on days 5 and 6 (for plants grown on $[K^+]_{ext} \leq 0.1$ mM) or on day 5 only (for all other growth conditions). A subset of experiments also involved plants grown on a nutrient-deprived solution of 200 µM CaSO$_4$ (see text). Plants were grown in walk-in growth chambers under fluorescent lights with an irradiation of ~200 µmol photons m$^{-2}$ s$^{-1}$ at plant height, for 16 h d$^{-1}$ (Philips Silhouette High Output F54T5/850HO; Philips Electronics Ltd, Markham, ON, Canada). Daytime temperature was 20 °C, night-time temperature was 15 °C, and relative humidity was approximately 70%.

Rice seeds (*Oryza sativa* L., cvs. ‘IR29’, ‘IR72’, and ‘Pokkali’) were surface-sterilized with 1% sodium hypochlorite for 10 min, germinated in aerated dH$_2$O for 48 h, and placed into 14-L plastic hydroponic vessels containing aerated, modified Johnson’s rice solution (2 mM MgSO$_4$, 0.3 mM NaH$_2$PO$_4$, 0.3 mM CaCl$_2$, 0.1 mM FeEDTA, 20 µM H$_3$BO$_3$, 9 µM MnCl$_2$, 1.5 µM CuSO$_4$, 1.5 µM ZnSO$_4$, 0.5 µM Na$_2$MoO$_4$), pH 6.30–6.35 (adjusted with 1 M NaOH). Potassium was supplied as K$_2$SO$_4$ at various concentrations (see text). Nitrogen was supplied either as Ca(NO$_3$)$_2$ or (NH$_4$)$_2$SO$_4$, at various concentrations (see text). Long-term salinity-stress treatments involved supplementation of the growth medium with 50 mM NaCl (see text). To ensure plants were maintained at a nutritional steady state, solutions were completely exchanged on days 9, 13, 16, 18, and 20 (post-sterilization), and were experimented with on day 21. Plants were cultured in climate-controlled, walk-in growth chambers under fluorescent lights with an irradiation of...
~400 µmol photons m$^{-2}$ s$^{-1}$ at plant height for 12 h d$^{-1}$ (Sylvania Cool White, 96T12/CW/VHO; Sylvania, Mississauga, ON, Canada). Daytime temperature was 30 °C, night-time temperature was 20 °C, and relative humidity was approximately 70%.

Seeds of Arabidopsis (*Arabidopsis thaliana*) wild-type ecotypes Col-0 (N1092) and WS (N1601) and t-DNA insertion lines *atakt1-1* (CS3762; WS ecotype), *athak5-1* (SALK_014177; Col-0 ecotype), and *athak5 atakt1* (Col-0 ecotype; Rubio et al. 2010) were surface-sterilized for 5 min with 70% ethanol, followed by 10 min with a 1% sodium hypochlorite-0.05% SDS mixture, and allowed to stratify in a 0.1% agar solution in the dark for 3 d at 4°C, prior to germination on acid-washed sand for 4 d. Seedlings were then placed in 14-L hydroponic vessels containing aerated nutrient solution composed of 5 mM K$_2$SO$_4$, 1 mM Ca(NO$_3$)$_2$, 1 mM NaH$_2$PO$_4$, 0.5 mM MgSO$_4$, 0.25 mM CaSO$_4$, 25 µM H$_3$BO$_3$, 20 µM FeEDTA, 2 µM ZnSO$_4$, 0.5 µM MnCl$_2$, 0.5 µM CuSO$_4$, and 0.5 µM Na$_2$MoO$_4$ (pH 6.0, with 1 M NaOH). Solutions were completely exchanged once per week for 3 weeks. During the final (5th) week of growth, [K$^+$]$_{ext}$ was typically changed (see text), and where N-source changes were made (see text), Ca(NO$_3$)$_2$ was replaced with an equimolar amount of (NH$_4$)$_2$SO$_4$. Nutrient solutions were completely exchanged every second day during the final week of growth and experimented with on day 35. This growth regime was particularly important for the proper growth of *atakt1* and *athak5 atakt1* mutants, as germination and growth were severely hindered on low-K$^+$, high-NH$_4^+$ media (data not shown; Rubio et al. 2010). Plants were grown in a climate-controlled chamber under fluorescent lights, with an irradiation of ~200 µmol photons m$^{-2}$ s$^{-1}$ at plant height, for 12 h d$^{-1}$ (Philips F96T8/TL841/HO/PLUS; Philips Electronics Ltd, Markham, ON, Canada). Daytime temperature was 20 °C, night-time temperature was 15 °C, and relative humidity was approximately 70%.

### 2.2 Radiotracer Analyses

#### 2.2.1 Radioisotopes

$^{42}$K (half-life = 12.36 h), was received as $^{42}$K$_2$CO$_3$ from the McMaster University Nuclear Reactor (Hamilton, ON, Canada). On the day of the experiment, ~0.2 g of $^{42}$K$_2$CO$_3$ (~40 mCi) was weighed out and dissolved in 19.93 mL dH$_2$O and 0.07 mL concentrated H$_2$SO$_4$, to drive the following chemical reaction:
The resulting $^{42}$K$_2$SO$_4$ solution was then used to “spike” loading solutions of various [K$^+$]$_{ext}$.

$^{13}$N (half-life = 9.97 min) was received as $^{13}$NH$_3$/$^{13}$NH$_4^+$ solution (~5 mL) from the Centre for Addiction and Mental Health (CAMH) cyclotron (University of Toronto, Toronto, ON, Canada). The tracer is produced by the proton bombardment of the oxygen atom of water (typically resulting in a radioactivity of 100-200 mCi; for production details, see Meeks 1993). Because the molar quantity of $^{14}$NH$_3$/$^{14}$NH$_4^+$ is extremely low in these solutions, the N concentration of the tracer stock solution was negligible. Thus, on the day of the experiment, a small aliquot (no more than ~0.5 mL) of stock solution was sufficient to “spike” loading solutions (Coskun et al. 2014a).

2.2.2 Direct Influx (DI)

One day before experimentation (day 6), barley seedlings were bundled together at the base of the shoot, in groups of three to four using a plastic collar, 0.5 cm in height. Bundling of multiple seedlings was done to ensure sufficient count retention during gamma counting (i.e. a high signal:noise ratio). There was no need to bundle Arabidopsis.

Unidirectional K$^+$ and NH$_3$/NH$_4^+$ influx in roots of intact barley and Arabidopsis was measured as described in detail elsewhere (Coskun et al., 2014a). In brief, roots were pre-equilibrated for 5 or 10 min (see text) in a solution either identical to the growth solution (control) or in growth solution supplemented with a chemical treatment (see below). Roots were then immersed for various times (see text) in a solution identical to the pre-equilibration solution but containing $^{42}$K$^+$ or $^{13}$NH$_3$/$^{13}$NH$_4^+$. From there, labeled plants were transferred to nonradioactive growth solution for 5 s to reduce tracer carryover and then further desorbed of radioactivity from the extracellular space for various amounts of time in fresh growth solution (see text). Immediately following desorption, roots were detached from shoots and spun in a low-speed centrifuge for 30 s to remove surface solution prior to weighing. Radioactivity in plant tissues was counted and corrected for isotopic decay using one of two $\gamma$ counters (Perkin-Elmer Wallac 1480 Wizard 3’; Perkin-Elmer, Waltham, MA, USA and Packard Instrument Quantum Cobra Series II, model 5003; Perkin-Elmer, Waltham, MA, USA). Throughout, influx is expressed in terms of $\mu$mol g$^{-1}$ (root fresh weight) h$^{-1}$. 
2.2.3 Compartmental Analysis by Tracer Efflux (CATE)

$^{42}$K$^+$ efflux from roots of intact barley and rice, and $^{13}$NH$_3$/1$^{13}$NH$_4^+$ efflux from roots of barley, were examined as described previously (Coskun et al., 2014a) and based on the method of compartmental analysis (Lee and Clarkson 1986; Siddiqi et al. 1991; Kronzucker et al. 1995). In brief, roots of replicate units of five to 10 bundled seedlings were labeled for 1 h in aerated nutrient media identical to growth conditions but containing $^{42}$K or $^{13}$N (see above). Labeled seedlings were then secured in glass efflux funnels, and roots were eluted of radioactivity with successive 20-mL aliquots of aerated, nonradioactive growth solution. The timing of each desorption series varied slightly among experiments (see text) but was typically as follows, from first to final eluate: 15 s (four times), 20 s (three times), 30 s (two times), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once), 2 min (13 times), for a total of 40 min of elution. Typically, the first 19 eluates (14 min into the elution series) were identical to the growth solution, while the final 13 eluates contained either growth solution (control) or a chemical treatment (see text).

Immediately following elution, plant organs were harvested as described in the preceding section, and radioactivity from eluates, roots, and shoots was counted and corrected for isotopic decay, as described above. For comparison charts of tracer efflux, the specific activities of all replicates were normalized to the arbitrary value of 2 x $10^5$ cpm µmol$^{-1}$. Throughout, tracer efflux was expressed in terms of cpm released g$^{-1}$ (root fresh weight) min$^{-1}$.

For the results of CATE (e.g. see Tables 1 and 7), tracer efflux and retention data were used to estimate unidirectional and net fluxes, cytosolic half-times of exchange, and cytosolic pool sizes, according to the methods of compartmental analysis (Kronzucker et al. 1995). In brief, linear regression of the function,

$$\ln \Phi_{co(t)}^* = \ln \Phi_{co(i)}^* - kt$$

(where $\Phi_{co(t)}^*$ is tracer efflux at elution time $t$, $\Phi_{co(i)}^*$ is initial tracer efflux, and $k$ is the rate constant describing the exponential decline in radioactive tracer efflux, found from the slope of the tracer release rate) was used to resolve the kinetics of the slowest exchanging (cytosolic) phase in these experiments (Kronzucker et al. 1995). For $^{42}$K$^+$, the cytosolic origin of the slowest exchanging phase was confirmed for the low-K$^+$ conditions (i.e. <1 mM [K$^+$]$_{ext}$; see Chapters 3
and 4), and ruled out for high-K⁺ conditions ([K⁺]₆ₓ ≥ 1 mM), by means of pharmacological testing (Coskun et al. 2010); thus, compartmental analysis of cytosolic pools and fluxes could not be performed at high K⁺. Based on literature precedents demonstrating the relatively long half-times of vacuolar K⁺ exchange compared with that of the cytosol (hours versus minutes; Poole 1971; Walker and Pitman 1976; Behl and Jeschke 1982; Memon et al. 1985; Hajibagheri et al. 1988; Siddiqi et al. 1991; White et al. 1991), we could assume that the vast majority of intracellular tracer released was from the cytosolic pool after only 1 h of loading, while vacuolar release was negligible. Chemical efflux, Φ₀, was determined from initial tracer efflux, Φ₀(i)*, divided by the specific activity of the cytosol (SAₖʸₜ) at the end of the labeling period. SAₖʸₜ was estimated by using external specific activity (SA₆ₓ), labeling time t, and the rate constant k, which are mathematically related in the exponential rise function (Walker and Pitman 1976),

\[ SA_{\text{cyt}} = SA_{\text{ext}} (1 - e^{-kt}) \]

Net flux, Φₙᵣ, was found using total plant (root and shoot) tracer retention after desorption, and influx, Φ₀ₑ, was calculated from the sum of Φ₀ and Φₙᵣ. Note that shoot accumulation of radiotracer is routinely dealt with in compartmental analysis of intact seedlings (Jeschke and Jambor 1981; Siddiqi et al. 1991); thus, the parameters listed in CATE Tables (e.g. Tables 1 and 7), can be accurately estimated. [K⁺]ₖʸₜ was determined using the flux turnover equation,

\[ [K^+]_{\text{cyt}} = \Omega \Phi_0 k^{-1} \]

where Ω is a proportionality constant correcting for cytosolic volume being approximately 5% of total tissue (Lee and Clarkson 1986; Siddiqi et al. 1991; Britto and Kronzucker 2001).

### 2.2.4 Pharmacological Treatments

For K⁺ influx experiments, the following agents were used to test for the involvement of different uptake mechanisms: 10 mM TEACl, 5 mM BaCl₂, 10 mM CsCl, 10 mM Na₃VO₄, 1 mM NaCN + SHAM, 100 µM DNP (1% ethanol), 50 µM DES (1% ethanol), 10 µM CCCP (1% ethanol), pH 9.2 (adjusted with 1 M NaOH), 5 mM CaSO₄, 5 mM CaCl₂ (1 mM in the case of Arabidopsis; see 4.1), 5 mM Ca(NO₃)₂ (1 mM for Arabidopsis), 50 µM GdCl₃, 50 µM LaCl₃, 10 mM sodium Glu, and 100 µM DEPC. All treatments involved a 5-min pretreatment, except for
DNP, DES, CN\(^-\) + SHAM, CCCP, and DEPC, which took 10 min. It should be noted that no effect of 1% ethanol (a solvent vehicle for DNP, DES, and CCCP) on K\(^+\) influx was found (data not shown). Inhibitors were added in the presence of a complete growth medium, unless stated otherwise.

For K\(^+\) efflux experiments, eluates were supplemented with 10 mM CsCl, 10 mM TEACl, 5 mM BaCl\(_2\), 10 mM LaCl\(_3\), 10 mM NaHCO\(_3\), 5 mM (NH\(_4\))\(_2\)SO\(_4\), NaCl (at various concentrations, see text), 5 mM Rb\(_2\)SO\(_4\), 5 mM K\(_2\)SO\(_4\), 5 mM Ca(NO\(_3\))\(_2\), 5 or 10 mM CaSO\(_4\) (see text), 10 mM Na\(_3\)VO\(_4\), pH 9.2 (adjusted with 1 M NaOH), 1 mM NaCN, 100 µM minoxidil sulphate (0.1% DMSO), 100 µM pinacidil monohydrate (0.1% DMSO), 100 µM diazoxide (0.1% DMSO), and 100 µM 4-aminopyridine (0.1% DMSO). Other treatments involved the withdrawal of (NH\(_4\))\(_2\)SO\(_4\) from growth solutions. A small subset of experiments also involved loading and elution solutions adjusted to a pH of 9.2 (with 1 M NaOH). The heavy-metal AQP inhibitors used in this study were Ag\(^+\) (as AgNO\(_3\)), Au\(^{3+}\) (as HAuCl\(_4\)), and Hg\(^{2+}\) (as HgCl\(_2\)), all provided at 5, 50, and 500 µM. A small subset of experiments also used 5 µM silver sulphadiazine (silver 4-amino-N-2-pyrimidinylbenzenesulphonamide, 0.5% DMSO; Niemietz and Tyerman, 2002). The organic AQP inhibitors acetazolamide and zonisamide (at 10 µM in 0.1% DMSO; Yool et al. 2010), and the AQP stimulator forskolin (at 10 µM in 0.1% DMSO; Maurel et al. 1995; Yool et al. 1996) were also tested. Control experiments showed no effect of either 0.1% or 0.5% DMSO on K\(^+\) fluxes (not shown). In addition, the heavy metals Cd\(^{2+}\) (as CdCl\(_2\)), Ni\(^{2+}\) (as NiSO\(_4\)), and Pb\(^{2+}\) (as Pb(NO\(_3\))\(_2\)), known to not generally inhibit AQPs (Niemietz and Tyerman 2002; see Zelenina et al. 2003; Verdoucq et al. 2008) were tested (all at 500 µM).

For \(^{13}\)N experiments, treatments included concentration-dependent and pH-dependent isotherms, whereby [NH\(_3$/NH\(_4^+$/ext and pH were adjusted (with NaOH) as specified (see text), in preloading and loading solutions. Other treatments included 10 mM CsCl, 5 mM K\(_2\)SO\(_4\), 10 mM NaCl, 10 mM ZnCl\(_2\), 500 µM HgCl\(_2\) and chilling (4 °C) in preloading (10 min) and loading solutions. A subset of experiments involved a 2-h pretreatment at pH 5.25 with or without N\(_2\) bubbling (anoxia treatment), 2 mM H\(_2\)O\(_2\), or 20 mM propionic acid.
2.3 Electrophysiology

Measurements of membrane potential ($\Delta \Psi_m$) from epidermal and cortical root cells of barley and Arabidopsis (aged 7–8 d and 35–37 d, respectively) were conducted as described previously (Schulze et al. 2012). In brief, roots were immersed in a Plexiglas chamber filled with nutrient solution and installed onto the stage of an inverted light microscope (Leica DME; Leica Microsystems, Richmond Hill, ON, Canada). Microelectrodes (tip diameter <1 µm), made from borosilicate glass (i.d. = 0.75 mm, o.d. = 1.00 mm; World Precision Instruments, Sarasota, FL, USA) and produced using an electrode puller (P-30; Sutter Instrument, Novato, CA, USA), were filled with 3 M KCl solution (pH 2). Both impaling and reference electrodes were prepared in this manner. $\Delta \Psi_m$ measurements were made in a region 2 to 3 cm from the root tip, with the use of an electrometer (Duo 773; World Precision Instruments, Sarasota, FL, USA), and recorded on an oscilloscope (TDS2002B; Tektronix, Markham, ON, Canada). Once steady readings were obtained, treatment solution was perfused through Tygon tubing via a peristaltic pump at a rate of approximately 7.5 mL min$^{-1}$ (see text for details regarding treatments). For thermodynamic (Nernstian) analyses, $E_{K^+}$ was estimated using the Nernst equation:

$$E_{K^+} = \frac{RT}{zF} \ln \frac{[K^+]_{ext}}{[K^+]_{cyt}}$$

where $R$ is the universal gas constant (8.314 J K$^{-1}$ mol$^{-1}$), $T$ is ambient temperature (293.15 K), $z$ is the ionic charge of the species (+1 for $K^+$), $F$ is the Faraday constant (96,485 C mol$^{-1}$), and $[K^+]_{ext}$ and $[K^+]_{cyt}$ are as defined previously.

2.4 Tissue-Content Analyses

2.4.1 Flame Photometry

Tissue $K^+$ and $Na^+$ contents for barley and rice (aged 7 and 21 d, respectively) were determined by methods described previously for steady-state and non-steady-state conditions (Britto et al. 2010; Coskun et al. 2012). In brief, replicate units of three to five seedlings had their roots incubated for 5 min in 10 mM CaSO$_4$ to release extracellular $K^+$ and $Na^+$ (steady state) or were treated in growth solution with NH$_4^+$ removed at various time points spanning 24 h (non-steady
state; see 4.1) prior to CaSO_4 desorption. From there, plant organs were harvested as above (see 2.2.2), oven dried for 3 d at 80-90 °C, and pulverized and digested in 30% HNO_3 for an additional 3 d. K\(^+\) and Na\(^+\) concentrations of tissue digests were measured using a dual-channel flame photometer (model 2655-10; Cole-Parmer Instrument, Montreal, QC, Canada). Tissue ion concentration was expressed in µmol g\(^{-1}\) (root fresh weight).

### 2.4.2 OPA Assay

Tissue NH\(_4^+\) content was determined by the o-phthalaldehyde (OPA) method as previously described (Szczerba et al. 2008b). Following desorption in 10 mM CaSO\(_4\) (see above), separation of organs, and weighing, root and shoot tissue (~0.5 g) were pulverized under liquid N\(_2\) using a mortar and pestle, and NH\(_4^+\) was extracted by addition of 6 mL of formic acid (10 mM) (Husted et al. 2000). Subsamples (1 mL) of the homogenate were centrifuged at 16,000 g at 2 °C for 10 min. Supernatants were transferred into 2 mL polypropylene tubes affixed with 0.45 µm nylon filters (Costar; Corning Inc., Lowell, MA, USA) and centrifuged at 5,000 g at 2 °C for 5 min. The resulting supernatant was analyzed using the OPA method to determine NH\(_4^+\) content (Goyal et al. 1988). OPA reagent was prepared 1 d before use and was composed of 100 mM KH\(_2\)PO\(_4\), 100 mM K\(_2\)HPO\(_4\), 3.75 mM OPA, and 2 mM 2-mercaptoethanol. Prior to the addition of 2-mercaptoethanol, the solution was adjusted to pH 7 with 1 M NaOH and filtered through grade-2 Whatman filter paper. A 10-µL aliquot of tissue extract was added to 3 mL of OPA reagent, and the colour was allowed to develop in the dark for 30 min at room temperature. Sample absorbance was measured at 410 nm using a spectrophotometer (Hewlett Packard 8453; Agilent Technologies Canada Inc., Mississauga, ON, Canada).

### 2.5 Root Respiration

Root respiration was determined by immersing roots of intact plants in a 3-mL cuvette containing nutrient solution, in which oxygen depletion was monitored by use of a Clark-type O\(_2\) electrode system (Hansatech Instruments Ltd., King’s Lynn, Norfolk, UK). The decline in dissolved O\(_2\) was recorded over 10 min, after which roots were dried and weighed, as described above. The theoretical respiratory cost of active ion efflux was determined using the relationship,
\[ \Phi_{O2} = 0.2 \Phi_{co} \]

where \( \Phi_{O2} \) represents the oxygen consumption by roots, and \( \Phi_{co} \) represents the efflux of monovalent cations (K\(^+\) or Na\(^+\)) from roots. This analysis is based on the electroneutral K\(^+\) (or Na\(^+\))/H\(^+\) exchange mechanism (see Subchapter 4.3), a stoichiometry of 1 H\(^+\) pumped per ATP hydrolysed (Britto and Kronzucker 2006), and a phosphorylation efficiency (P/O\(_2\) ratio) of 5 (Kurimoto et al. 2004; Britto and Kronzucker 2006).

### 2.6 Confocal Microscopy

Following a 15-min treatment with Ag\(^+\), Au\(^{3+}\), Hg\(^{2+}\), Pb\(^{2+}\), Cd\(^{2+}\), Ni\(^{2+}\), and Na\(^+\) (see text for details), lateral root segments of intact seedlings were sectioned 0.5–1 cm from the apex and incubated with 25 \( \mu \)g mL\(^{-1}\) propidium iodide with 0.1% Silwet to counterstain the cell wall and nuclei of ruptured cells (Oh et al. 2010). Propidium iodide was excited at 488 nm and fluorescence was detected at \( \geq 585 \) nm (red channel) using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

### 2.7 Membrane-Stability Assays

#### 2.7.1 Electrical Conductivity

Electrolyte leakage, as a measure of membrane intactness, was assessed as described by Tuna et al. (2007) but adapted here for excised roots. After a 15-min incubation in either normal growth solution (control) or growth solution plus 500 \( \mu \)M AgNO\(_3\), roots (~150 mg) of intact seedlings were excised and rinsed three times with deionized water to remove surface-adhering electrolytes and damaged cells from the cut surface. Root segments were placed in 20 mL vials containing 10 mL of deionized water and incubated at 25 °C for 24 h in a rotary shaker. Subsequently, electrical conductivity (EC) of the bathing medium was measured (CON510 Bench Conductivity/TDS meter; Oakton Instruments, Vernon Hills, IL, USA). After samples were autoclaved at 120 °C for 20 min, the final electrical conductivity of the bathing medium was measured following equilibration at 25 °C. The relative leakage ratio was determined as the quotient of the EC before and after autoclaving.
2.7.2 UV-Absorbance Spectrophotometry

Another gauge of membrane stability involved measuring the release of UV-absorbing substances from excised roots (Redmann et al. 1986). Roots were treated as described above but absorbance (at 280 nm, $A_{280}$) of the bathing medium was measured spectrophotometrically (see above). The relative leakage ratio was determined as the quotient of $A_{280}$ before and after autoclaving.

2.8 Ag$^+$-Induced K$^+$-Release Quantification

In efflux experiments, silver stimulated radiotracer release from roots over the course of its application (see Chapter 5). To express this release in terms of moles of traced ions, it was essential to estimate the specific activity (SA) of the tracer pool captured in efflux aliquots. This was done by first determining cytosolic SA following the 1-h labelling period, using the exponential rise function described above (see 2.2.3). Cytosolic SA, however, had to be corrected for $^{42}$K$^+$ lost during the first 15.5 min of efflux desorption ($i.e.$ prior to silver application), using the exponential decay function,

$$SA_{cyt}' = SA_{cyt} e^{-kt'}$$

or by substitution in the equation,

$$SA_{cyt}' = SA_{ext} (1 - e^{-kt}) e^{-kt'}$$

where $t' = 15.5$ min. Thus, to quantify the K$^+$ released (in µmol g$^{-1}$ (root FW)) after silver application, the radioactivity released (in cpm) during this time was summed, divided by $SA_{cyt}'$, and corrected for root FW. For comparison purposes, this protocol was conducted for each individual treatment on efflux.

2.9 Root Water Potential, $\Psi_R$

Water potential ($\Psi_R$) of control roots or roots treated with either 500 µM AgNO$_3$ or HAuCl$_4$ was measured by means of the pressure chamber (Model 1000 Pressure Chamber Instrument; PMS
Instrument Co., Albany, NY, USA) (Scholander et al. 1965). Following a 15-min incubation in the chemical treatment of interest, seedlings were detopped at the hypocotyl region (~1– 2 cm above the seed) and the root system was placed into the pressure chamber, allowing ~0.5 cm of the stem to protrude from the chamber. Root water potential (in MPa) was determined by applying N₂ gas pressure to the root system until the meniscus reached the cut surface.

2.10 PTS Efflux

The fluorescent dye 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS; Yeo et al. 1987) was used to trace apoplastic fluxes in roots of intact barley seedlings grown in a full nutrient solution supplemented with 0.1 mM K⁺ (see above). Roots of replicate units of 6–10 plants were immersed in growth solution supplemented with 0.01% (w/v) PTS for 1 h. From there, plants were transferred and secured into efflux funnels (see above), and eluted with successive 13-mL aliquots of fresh, PTS-free, growth solution over the course of 30 min. The elution series was as follows: 1 min (five times), 2 min (once), 3 min (once), 4 min (four times). From there, plants were harvested as mentioned above, and PTS fluorescence in eluates, roots, and shoots was measured by fluorimetry (excitation = 403 nm and emission = 510 nm; BioTek Synergy 4; BioTek, Winooski, VT, USA).
Chapter 3
K⁺ Efflux in Roots of Higher Plants: Mechanisms and Connections to Abiotic Stress

3.1 Regulation and Mechanism of Potassium Release from Barley Roots: An in planta ⁴²K Analysis

Preface


HJ Kronzucker designed the study, with input from DT Britto and D Coskun. D Coskun performed the experiments and analyzed the data, with input from DT Britto and HJ Kronzucker. DT Britto wrote the manuscript, with input and editing from D Coskun and HJ Kronzucker.

3.1.1 Abstract

Potassium (K⁺) flux into plant cells is a well-characterized ion transport phenomenon. By contrast, little is known about the mechanisms and regulation of K⁺ flux from the cell. Here, we present a radioisotopic analysis of K⁺ fluxes from roots of intact barley (Hordeum vulgare), in the context of recent discoveries in the molecular biology and electrophysiology of this process. Plants were labelled with ⁴²K⁺, and kinetics of its release from roots were monitored at low (0.1 mM) or high (1.0 mM) external K⁺ concentration, [K⁺]ₐₐ₁, and with the application of channel modulators and nutrient shifts. At 0.1 (but not 1.0) mM [K⁺]ₐₐ₁, where K⁺ efflux is thought to be mediated by K⁺-outward-rectifying channels, ⁴²K⁺ efflux was inhibited by the channel blockers barium (Ba²⁺), cesium (Cs⁺), tetraethylammonium (TEA⁺), and lanthanum (La³⁺). Ammonium and nitrate (10 mM) stimulated and inhibited ⁴²K⁺ efflux, respectively, while 10 mM [K⁺]ₐₐ₁ or [Rb⁺]ₐₐ₁ decreased it. No evidence for the involvement of ATP-binding cassettes, nonselective cation channels, or active K⁺-efflux pumps was found. Our study provides new evidence for the thermodynamic transition between high- and low-affinity transport, from the efflux perspective,
identifying the operation of channels at low $[K^+]_{\text{ext}}$, and the cessation of transmembrane efflux at high $[K^+]_{\text{ext}}$.

3.1.2 Introduction

Potassium ($K^+$), a major macronutrient, is the most abundant cation in plant cells, and is required for a wide range of functions including osmotic balance, electrical regulation, and enzyme activation. Its uptake by plant roots has been studied extensively for decades, and a robust two-system model is widely used that describes the distinct kinetics of $K^+$ acquisition at low external $K^+$ concentrations ($[K^+]_{\text{ext}}$; a ‘high-affinity’ system) and at higher external concentrations (a ‘low-affinity’ system), the demarcation between the two typically falling in the range of 0.5–1.0 mM $[K^+]_{\text{ext}}$ (Britto and Kronzucker 2008). This model was initially developed on the basis of uptake kinetics, as measured by radiotracers, particularly in the pioneering work of Epstein (e.g. Epstein et al. 1963). The two systems differ from each other in a number of fundamental ways, including their regulation, thermodynamics, saturation kinetics and response to nitrogen source (for a review, see Britto and Kronzucker 2008).

More recently, specific transport proteins have been identified that catalyse $K^+$ fluxes in both the high- and low-affinity ranges. The saturable, high-affinity system has been linked to the HAK/KUP/KT family of transporters, while the linear, low-affinity system is associated with the activity of inwardly-rectifying $K^+$-specific ion channels. The molecular physiology of these transporters has been discussed in several recent reviews (including Véry and Sentenac 2003; Gierth and Mäser 2007; Lebaudy et al. 2007; Dreyer and Blatt 2009; Szczerba et al. 2009). It should be pointed out that there is some overlap in function between these two types of transporters, for example with AtKUP1 from Arabidopsis catalysing some degree of transport in the low-affinity range (Fu and Luan 1998; Kim et al. 1998), and the Arabidopsis AKT1 channel conducting $K^+$ fluxes in the high-affinity range (Hirsch et al. 1998). However, because channel-mediated transport takes place passively, AKT1 can only facilitate $K^+$ fluxes in the high-affinity range when the electrical state of the plasma membrane is highly polarized.

In contrast to the influx of $K^+$, little is known about the physiology and molecular biology of $K^+$ efflux from plant roots. Physiological studies examining the efflux of $K^+$ have largely been
restricted to two areas: compartmental analysis, in which efflux kinetics are used to determine subcellular pool sizes as well as other fluxes such as influx into the cell, and fluxes to the vacuole and shoot (Pitman and Saddler 1967; Macklon 1975; Memon et al. 1985; Kronzucker et al. 2003); and the analysis of K⁺ loss from roots in response to salt or ionic stress (Nocito et al. 2002; Shabala et al. 2006; Britto et al. 2010). Similarly, the molecular biology of K⁺ release from the root is poorly understood, although several channels involved in the release of K⁺ from root cells have been identified. These include SKOR, which facilitates K⁺ release into the xylem from neighbouring parenchyma cells (Gaymard et al. 1998), and GORK, which normally functions in K⁺ release from guard cells in leaf tissue, but has also been localized in root hairs of Arabidopsis (Ivashikina et al. 2001). In addition, assembly of AKT1 homotetramers in Arabidopsis mutants lacking the accessory channel protein AtKC1 can result in the formation of functional channels that conduct ‘leak’ fluxes from the cell (Geiger et al. 2009). However, SKOR and GORK are not known to mediate K⁺ efflux from the root to the external environment, and AKT1 homotetramers are the products of mutagenesis and do not occur naturally. Thus, the molecular identity of ion channels mediating K⁺ loss from the plant root into the external environment, like their physiological characteristics, remains largely unknown. Further complicating the issue is the apparent diversity of outwardly directed channel activity among plant species and cell type (Diatloff et al. 2004). In addition to leaving the cell via ion channels, K⁺ may also be driven outwardly via cation/proton exchangers such as those of the CHX (cation-proton exchanger) family (Pardo et al. 2006), although the direction of the flux catalysed by these exchangers has been recently questioned (Zhao et al. 2008).

In the present study, we have sought to address the lack of knowledge about K⁺ efflux from plant roots, by using radiotracer analysis to examine in detail its physiological properties in intact barley seedlings. In particular, we have posed the following questions: What mechanisms underlie and regulate K⁺ efflux from roots to the external environment? How does K⁺ efflux respond to shifts in external [K⁺] from high- to low-affinity conditions? How do the principal nitrogen sources for plants (ammonium and nitrate) differ in their effects upon K⁺ efflux? Throughout, we have sought to answer these questions as a means of augmenting emerging discoveries in the molecular biology and electrophysiology of K⁺ efflux.
3.1.3 Results

Unidirectional and net fluxes in control, steady-state plants grown at 0.1 or 1.0 mM $[\text{K}^+]_{\text{ext}}$ were determined using tracer efflux and retention data, and are shown in Table 1. Efflux, influx and the ratio of the two were all lower in the high-K$^+$ plants, while the net flux of K$^+$ was nearly the same under both conditions.

Fig. 2 shows the effects of channel-blocking agents on the efflux of $^{42}\text{K}^+$ from pre-labelled roots of intact barley seedlings, grown at two concentrations of K$^+$. At low $[\text{K}^+]_{\text{ext}}$ (0.1 mM), $^{42}\text{K}^+$ efflux was reduced by application of TEA$^+$ and La$^{3+}$, and blocked by Ba$^{2+}$ and Cs$^+$ (Fig. 2). Because of the greater efficacy of Ba$^{2+}$ and Cs$^+$ at 0.1 mM, these agents were used for efflux trials at higher $[\text{K}^+]_{\text{ext}}$. Nevertheless, we found that neither agent changed the efflux pattern at 1.0 mM (Fig. 2, inset; a small suppressive effect of Ba$^{2+}$ was seen at 0.5 mM, not shown).

In addition to its response to channel blockers, $^{42}\text{K}^+$ efflux from roots of 0.1 mM-grown plants was also suppressed by the application of high concentrations (10 mM) of potassium or rubidium, while neither treatment altered efflux from plants grown at 1.0 mM (Fig. 3). In plants grown at 0.1 mM, application of 10 mM Ca$^{2+}$ or 10–25 mM sodium had no effect on $^{42}\text{K}^+$ efflux, but efflux rose when 100 mM sodium was applied (Fig. 3A and B).

Nitrogen source had an immediate effect on $^{42}\text{K}^+$ efflux, at both low (0.1 mM) and high (1.0 mM) K$^+$ provision (Fig. 4). All plants, grown at 1 mM NO$_3^-$, responded to the addition of 10 mM NH$_4^+$ with an approximately threefold increase in $^{42}\text{K}^+$ efflux. Application of 10 mM NH$_4$NO$_3$ also accelerated $^{42}\text{K}^+$ efflux, but to a lesser degree than 10 mM NH$_4^+$. Similar responses to high NH$_4^+$ and NH$_4$NO$_3$ were seen at the intermediate $[\text{K}^+]_{\text{ext}}$ values of 0.5 mM and 0.75 mM (not shown). The shape of the ammonium-stimulated $^{42}\text{K}^+$ efflux trace differed between the 0.1 mM $[\text{K}^+]_{\text{ext}}$ treatment and the others, however, in that at 0.1 mM it peaked within 2 min, then declined (Fig. 4A). By contrast, $^{42}\text{K}^+$ efflux at higher $[\text{K}^+]_{\text{ext}}$ rose more slowly and reached a plateau at which it was sustained for at least 10 min (Fig. 4B). Another key difference between low and high K$^+$ conditions was that, at 0.1 $[\text{K}^+]_{\text{ext}}$, the application of 10 mM NO$_3^-$ suppressed $^{42}\text{K}^+$ efflux (Fig. 4A), while this treatment had no effect at 1.0 mM $[\text{K}^+]_{\text{ext}}$ (Fig. 4B).

Stimulation of $^{42}\text{K}^+$ efflux by NH$_4^+$ was ameliorated by the application of channel-blocking agents, but the efficacy of each agent depended on external $[\text{K}^+]$ conditions (insets, Fig. 4A and
B). At 0.1 mM \([K^+]_{ext}\), Cs\(^+\) was the most potent, reducing efflux to below control levels (as in Fig. 2A), while TEA\(^+\) was nearly as effective (Fig. 4A, inset). By contrast, at 1.0 mM \([K^+]_{ext}\), Cs\(^+\) and TEA\(^+\) treatments showed a moderate degree of suppression (Fig. 4B, inset). Interestingly, application of K\(^+\) itself (at 10 mM) also suppressed the NH\(_4^+\)-stimulated \(^{42}\)K\(^+\) efflux, as strongly as Cs\(^+\) under the 0.1 mM \([K^+]_{ext}\) conditions, and more moderately (comparable to TEA\(^+\) and Cs\(^+\)) under the 1.0 mM condition (not shown). In addition, application of 10 mM Rb\(^+\) was nearly as effective as 10 mM K\(^+\) in both high- and low-K\(^+\)-grown plants.

Direct influx measurements, complementary to efflux runs, were also conducted in relation to nitrogen source and strength (Fig. 5; values were slightly different from those determined using efflux analysis, but showed similar trends). Switching from the growth concentration of 1 mM NO\(_3^-\) to 10 mM NO\(_3^-\) had opposite effects on low and high-K\(^+\) plants, with elevated NO\(_3^-\) decreasing influx by c. 20% in low-K\(^+\) plants, and increasing it in high-K\(^+\) plants by c. 70%. Both NH\(_4^+\) and NH\(_4\)NO\(_3\), provided at 10 mM, decreased influx under both growing conditions.

It was of interest to investigate the effect on \(^{42}\)K\(^+\) efflux of treatments known to disrupt the energy state of the cell and the plasma membrane. Fig. 6 shows that the metabolic inhibitors vanadate (VO\(_4^{3-}\)) and cyanide (CN\(^-\)) increased \(^{42}\)K\(^+\) efflux at 0.1 mM \([K^+]_{ext}\), but had little or no effect at the higher \([K^+]_{ext}\) (1.0 mM, Fig. 6, inset). The addition of sodium bicarbonate, which is known to hyperpolarize the plasma membrane (Poole 1969), at 10 mM caused a suppression of \(^{42}\)K\(^+\) efflux at the lower \([K^+]_{ext}\) (0.1 mM) but not at the higher \([K^+]_{ext}\) (1.0 mM), while increasing the external pH from 6.3–6.5 to 9.2 had no effect at either \([K^+]_{ext}\).
Figure 2. Response of $^{42}$K$^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of the channel inhibitors Cs$^+$ (as CsCl), Ba$^{2+}$ (as BaCl$_2$), TEA$^+$ (as tetraethylammonium-Cl), or La$^{3+}$ (as LaCl$_3$) at external K$^+$ concentration, [$K^+$]$_{ext}$, of 0.1 mM. Inset: response of $^{42}$K$^+$ efflux to channel inhibitors Cs$^+$ and Ba$^{2+}$ at [$K^+$]$_{ext}$ of 1.0 mM. For clarity, data points within the inset were connected with coloured lines. In internal legend, numbers in parentheses following treatment conditions indicate per cent of treated points differing significantly from control ($t$-test, $P < 0.05$). Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.
**Figure 3.** Response of $^{42}$K$^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden application or elevation (at elution time = 15.5 min; see arrow) of Ca$^{2+}$ (as CaSO$_4$), K$^+$ (as K$_2$SO$_4$), Rb$^+$ (as Rb$_2$SO$_4$) or Na$^+$ (as NaCl). Growth concentrations of K$^+$ were 0.1 mM (A, B) or 1.0 mM (A, inset). Internal legend, as in Fig. 2. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.
Figure 4. Response of $^{42}\text{K}^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of NH$_4^+$ (as (NH$_4$)$_2$SO$_4$), NH$_4$NO$_3$, or NO$_3^-$ (as Ca(NO$_3$)$_2$). Inset: response of $^{42}\text{K}^+$ efflux to sudden provision of NH$_4^+$ in combination with the channel inhibitors TEA$^+$ (as TEACL) or Cs$^+$ (as CsCl). Plants were grown at an external K$^+$ concentration, [$K^+$]$_{ext}$, of 0.1 mM (A) or 1.0 mM (B). Internal legend, as in Fig. 2. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.
Figure 5. Direct $K^+$ influx determined by short-term (5 min) $^{42}K^+$ labelling of intact barley ($Hordeum vulgare$) seedlings, grown at either 0.1 or 1.0 mM (inset) external K concentration, $[K^+]_{ext}$, and introduced to sudden provision (10 min) of elevated $NO_3^-$ (as $Ca(NO_3)_2$), $NH_4^+$ (as $(NH_4)_2SO_4$), or $NH_4NO_3$. Letters indicate significantly different means (one-way ANOVA with Bonferroni post-test, $P < 0.05$). Error bars indicate ± SEM.
Figure 6. Response of $^{42}$K$^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of VO$_4^{3-}$ (as Na$_3$VO$_4$), CN$^-$ (as NaCN), pH 9.2, or HCO$_3^-$ (as NaHCO$_3$) at an external K concentration, [K$^+$]$_{ext}$, of 0.1 mM and 1.0 mM (inset). Internal legend, as in Fig. 2. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.
Table 1. Fluxes and compartmentation of K⁺ in roots of barley seedlings grown at either 0.1 or 1.0 mM [K⁺]_{ext}, as determined by compartmental analysis by tracer efflux (CATE).

<table>
<thead>
<tr>
<th>[K⁺]_{ext} (mM)</th>
<th>K⁺ influx (µmol g⁻¹ (root FW) h⁻¹)</th>
<th>K⁺ efflux (µmol g⁻¹ (root FW) h⁻¹)</th>
<th>Net K⁺ flux (µmol g⁻¹ (root FW) h⁻¹)</th>
<th>Efflux:Influx ratio</th>
<th>Cytosolic [K⁺] (mM)</th>
<th>Half-time of exchange (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>7.22 ± 0.23</td>
<td>1.86 ± 0.18</td>
<td>5.36 ± 0.18</td>
<td>0.25 ± 0.02</td>
<td>98.84 ± 14.08</td>
<td>28.18 ± 3.40</td>
</tr>
<tr>
<td>1.0*</td>
<td>5.97 ± 0.77</td>
<td>0.57 ± 0.03</td>
<td>5.40 ± 0.77</td>
<td>0.11 ± 0.01</td>
<td>53.14 ± 9.80</td>
<td>17.56 ± 1.36</td>
</tr>
</tbody>
</table>

3.1.4 Discussion

Although $^{42}\text{K}^+$ efflux patterns (Fig. 2) and flux parameters (Table 1) were similar among all control plants, regardless of growth condition, treatment of roots with a wide variety of physiologically active agents belies these similarities. With rising $\text{K}^+$ provision, the efficacy of treatments inhibiting $^{42}\text{K}^+$ efflux at the lowest $[\text{K}^+]_{\text{ext}}$ of 0.1 mM was progressively reduced (not shown), until there was very little effect at the highest $[\text{K}^+]_{\text{ext}}$ of 1.0 mM. When comparing the bracketing conditions of 0.1 and 1.0 mM $[\text{K}^+]_{\text{ext}}$ (referred to subsequently as ‘low-$\text{K}^+$’ and ‘high-$\text{K}^+$’, respectively), the difference is clear as the following six treatments had effects on $^{42}\text{K}^+$ efflux under low $\text{K}^+$-growth conditions, but none under high $\text{K}^+$: Cs$^+$, Ba$^{2+}$ (Fig. 2), K$^+$ (stepped up to 10 mM), Rb$^+$ (Fig. 3), NO$_3^-$ (Fig. 4) and HCO$_3^-$ (Fig. 6). In addition, treatment with the metabolic inhibitors cyanide and vanadate resulted in immediate stimulation of $^{42}\text{K}^+$ efflux at low $\text{K}^+$, but only a weak stimulation at high $\text{K}^+$ (Fig. 6).

It is reasonable to conclude from these results that, despite their superficial similarities, the efflux traces under low-$\text{K}^+$ and high-$\text{K}^+$ growth conditions represent two distinct phenomena. This idea is supported by substantial work indicating that the transition between high- and low-affinity $\text{K}^+$ transport modes in plant roots typically lies between 0.1 and 1.0 mM $[\text{K}^+]_{\text{ext}}$ (Epstein et al. 1963; Glass and Dunlop 1978; Kochian and Lucas 1982; Maathuis and Sanders 1995; Kronzucker et al. 2003). Over this range, the thermodynamic conditions change owing to $\text{K}^-$-induced alterations in the electrical potential difference across the plasma membrane (Etherton and Higinbotham 1960; Cheeseman and Hanson 1979a, b; Kochian et al. 1989; Walker et al. 1996b; Szczerba et al. 2006), as well as to changes in the concentration (activity) ratio of $\text{K}^+$ across this membrane. The result is a shift in the mechanism of $\text{K}^+$ influx into the cell, from an energetically active system to one catalysing transport down an electrochemical potential gradient as $[\text{K}^+]_{\text{ext}}$ increases. It is to be expected, then, that the opposite thermodynamic conditions will apply to the transport of $\text{K}^+$ in the efflux direction, from the cell to the external medium. Because many passive transport functions in biological systems are mediated by ion channels, it is reasonable to suggest that the $^{42}\text{K}^+$ efflux traces at 0.1 mM $[\text{K}^+]_{\text{ext}}$ represent efflux of $\text{K}^+$ from the cell down its electrochemical potential gradient, via outwardly-rectifying, Shaker-type $\text{K}^+$ (KOR) channels that have been identified at a molecular level and have been localized to the plasma membrane (for a review, see Szczerba et al. 2009). This idea is supported by the efficacy of ‘classic’ $\text{K}^+$-channel-blocking agents TEA$^+$, La$^{3+}$, Ba$^{2+}$ and Cs$^+$ (Krol and Trebacz 2000; White and Broadley 2000) in reducing
$^{42}$K$^+$ efflux at low [K$^+$]$_{ext}$, as depicted in Fig. 2. This inhibition profile parallels that of inward-rectifying, Shaker-type K$^+$ (KIR) channels (White and Broadley 2000), and agrees with the study by Roberts and Tester (1995), who showed that TEA$^+$ is not as effective as Cs$^+$ or Ba$^{2+}$ in blocking outward, whole-cell, K$^+$ currents in maize root protoplasts. Similarly, the observation (Fig. 3A) that K$^+$ and Rb$^+$ (when either was provided at 10 mM) suppressed $^{42}$K$^+$ efflux in plants grown at 0.1 mM [K$^+$]$_{ext}$ supports KOR channel involvement, as K$^+$ efflux channels in plants, rather uniquely, are known to be inhibited by elevated external K$^+$ or Rb$^+$, through cooperative binding of these ions at regulatory sites distinct from the channel pore (Blatt 1999; Johansson et al. 2006; Gajdanowicz et al. 2009). Importantly, the observation that Rb$^+$ inhibited $^{42}$K$^+$ efflux to the same extent as elevated K$^+$ indicates that it is not the transmembrane concentration gradient of K$^+$ that brings about this channel closure, but more likely the regulatory binding of K$^+$, or its analogue Rb$^+$.

Interestingly, the application of high (10 mM) external Ca$^{2+}$ had no effect at 0.1 [K$^+$]$_{ext}$ (Fig. 3), in agreement with the relative Ca$^{2+}$-insensitivity of K$^+$ channels seen in most electrophysiological studies (White and Broadley 2000), but in contrast to one class of nonselective, outward-rectifying channels (NORCs), a hallmark of which is inhibition by low to intermediate concentrations of Ca$^{2+}$ (Demidchik and Maathuis 2007). The lack of NORC involvement in the present study is further supported by the insensitivity to Cs$^+$ and TEA$^+$ of all classes of NORCs (White and Broadley 2000; Demidchik and Maathuis 2007).

The number of molecular candidates that could account for the observed flux patterns at 0.1 mM [K$^+$]$_{ext}$ is limited. While several outward-rectifying K$^+$ channels have been identified at the molecular level in plant roots, none matches the $^{42}$K$^+$ efflux profiles reported here. The SKOR protein, while having an inhibition and gating profile suggestive of the present observations (Gaymard et al. 1998; Johansson et al. 2006), is an unlikely match because it is localized to pericycle and xylem parenchyma cells, consistent with its proposed role as a xylem-loading transporter. Owing to the immature Casparian strip in these young seedlings, however, it is possible that at least some of the $^{42}$K$^+$ is released from stelar cells and may diffuse to the external medium via the apoplast. The guard-cell channel GORK, while predominantly expressed in leaves, has also been detected in epidermal root hairs, but was found to be unaffected by 10 mM Cs$^+$ (Ivashikina et al. 2001), thus also disqualifying it as a major conductor of the observed flux in the present study. Similarly, Diatloff et al. (2004) examined outward-rectifying channels in
epidermal cells from roots of *Arabidopsis thaliana*, but these displayed a reduction in current density as extracellular K⁺ activity was reduced, contrary to the present findings. In addition, guard-cell outward rectifiers have been shown to be ATP-regulated via ATP-binding cassettes (ABC) (Leonhardt et al. 1997), but in our study, several chemical agents known to either stimulate or inhibit the activity of ABC proteins had little or no effect on ⁴²K⁺ efflux (not shown). Finally, the KCO1 channel, which has a pharmacological profile similar to the fluxes in the present study, is localized in the tonoplast and appears only to be activated episodically, in response to transient changes in cytosolic Ca²⁺ (Czempinski et al. 2002). In summary, our results suggest that the transport system dominating the mediation of efflux of K⁺ from roots of plants grown in low K⁺ has not as yet been identified at the molecular level. We further suggest that this study should complement and guide molecular and physiological K⁺ transport studies, which are typically performed at a less intact level.

Unlike the effects observed at 0.1 mM [K⁺]₆ₓ, ⁴²K⁺ efflux from plants grown at 1.0 mM was scarcely altered by the application of channel blockers (Fig. 2) or by elevated external K⁺ or Rb⁺ (not shown). In addition, the reduction of ⁴²K⁺ efflux observed upon application of elevated (10 mM) NO₃⁻ to roots of plants grown at 0.1 mM [K⁺]₆ₓ was not seen in plants grown at 1.0 mM [K⁺]₆ₓ (Fig. 4A and B). Thus, our data indicate that channel-mediated K⁺ efflux is likely to be inoperative under the high-K⁺ conditions. This is consistent with the conclusion drawn from patch-clamping studies that show KOR channels to be gated closed, via sensing of both external [K⁺] and membrane voltage, at membrane potentials more positive than the Nernst potential for K⁺ (Roberts and Tester 1995; Dreyer and Blatt 2009; Gajdanowicz et al. 2009).

Under the higher-K⁺ growth regime, the resistance of ⁴²K⁺ efflux traces to multiple inhibitors raises the question of how to interpret the observed ⁴²K⁺ release. To test the hypothesis that these traces represent ⁴²K⁺ driven from the cell via secondary active transporters (e.g. K⁺/H⁺ antiporters; Szczerba et al. 2006), as has been proposed for members of the CHX family (Pardo et al. 2006), we decreased the external [H⁺] by almost three orders of magnitude and applied the metabolic disruptors cyanide and vanadate. However, none of these treatments could decrease the flux (Fig. 6, inset). In addition, the role of CHX proteins in K⁺ transport is unclear, with one recent report indicating that at least one member of this family (the Arabidopsis AtCHX13) plays a role in K⁺ acquisition, rather than in its loss (Zhao et al. 2008). Furthermore, while membrane pumps catalysing ATP-driven, monovalent cation efflux from the cell have been found in
organisms such as yeast (Benito et al. 2002) and bryophytes (Benito and Rodriguez-Navarro 2003), there is no evidence that they exist in higher plants. Thus, our study suggests that the ostensibly channel-mediated efflux system observed at 0.1 mM [K+]_{ext} is not likely to be operating at 1.0 mM, nor are K⁺-efflux pumps. Rather, the evidence suggests that the ^{42}K⁺ released under these conditions crosses no membrane. As such, this observation is reminiscent of the phenomenon of apoplastic bypass flow, which has been documented in the case of sodium transport in cereals (Oertli 1968; Flowers et al. 1991; Gong et al. 2006; Malagoli et al. 2008; Krishnamurthy et al. 2009). If the observed ^{42}K⁺ traces at 1.0 mM [K⁺]_{ext} are indeed the result of extracellular K⁺ fluxes, this raises an important issue about cellular flux analysis by use of tracers: it is not always possible to distinguish components of the measured flux across the plasma membrane from those that occur outside the cell, as discussed by Cheeseman (1986). It is of further interest that, in Table 1, the calculated chemical efflux of K⁺ at 1.0 mM [K⁺]_{ext} is less than one-third that of the efflux calculated at 0.1 mM [K⁺]_{ext}, an unexpected outcome given that, over broader concentration ranges, efflux tends to rise (Britto and Kronzucker 2006) for most ions, including potassium (Szczerba et al. 2006). This provides further evidence that a different phenomenon is occurring at 1.0 mM [K⁺]_{ext}, which is reflective of the cessation of physiological efflux across the plasma membrane and the observation of efflux from the apoplast. It should also be pointed out that, even in the 0.1 mM [K⁺]_{ext} condition, a small proportion of the observed efflux may also be apoplastic. However, this contribution will amount to no more than 10% of the relatively low efflux seen at 1.0 mM, assuming linearity of this component with respect to [K⁺]_{ext}. Nevertheless, this additional efflux component may dominate tracer release kinetics at higher concentrations (e.g. those used in studies of salinity stress), wherein efflux becomes a sizeable proportion of the total flux (Britto and Kronzucker 2006; Malagoli et al. 2008). It should be pointed out that other possibilities, such as vesicular transport, cannot be ruled out (Peiter et al. 2007; Britto and Kronzucker 2009). Further research will be required to explore this possibility.

It has long been known that inorganic nitrogen source strongly influences the uptake of K⁺ by plant roots, particularly in the high-affinity range, over which K⁺ uptake is inhibited by NH₄⁺ (Vale et al. 1988a; Santa-María et al. 2000; Britto and Kronzucker 2002; Szczerba et al. 2008a). In addition, it is known that the co-presence of NO₃⁻ and K⁺ enhances the uptake of both ions (Blevins et al. 1974; Vale et al. 1988b). However, the impact of different N sources on root K⁺
efflux has been unexplored, apart from a few studies conducted under steady-state conditions (Rygiewicz and Bledsoe 1986; Kronzucker et al. 2003; Szczepański et al. 2005). In the present study, we found that changing N source and strength during tracer elution from 42K⁺-labelled barley roots had pronounced effects on the pattern of efflux (Fig. 4). In particular, the addition of 10 mM NH₄⁺ alone, or 10 mM NH₄NO₃, accelerated 42K⁺ efflux at both 0.1 mM [K⁺]ₜₐₓ and 1.0 mM [K⁺]ₜₐₓ. Indeed, these were the only treatments that affected efflux at 1.0 mM. These results suggest, along with the effects brought about by the co-provision, with NH₄⁺, of Cs⁺, TEA⁺ (Fig. 4A and B) and elevated K⁺ and Rb⁺ (not shown), that the introduction of high NH₄⁺ to high-K⁺ plants triggers the activity of a physiologically responsive, channel-mediated efflux. The incomplete inhibition of NH₄⁺-enhanced 42K⁺ efflux by these agents, relative to the NH₄⁺-free conditions (Fig. 2), might be explained by the opening, by NH₄⁺, of additional channels with different pharmacological profiles, but which are silent under NH₄⁺-free conditions; this idea warrants further investigation.

The simplest explanation for the general NH₄⁺-stimulated elevation of 42K⁺, under both low-K⁺ and high-K⁺ conditions, is the depolarization of the plasma membrane of root cells, which is known to occur when ammonium is applied (Ullrich et al. 1984; Ayling 1993; Wang et al. 1994; Nocito et al. 2002). This would shift the thermodynamic condition in favour of passive K⁺ efflux in the case of the high-K⁺ plants, and enhance the already favourable conditions driving K⁺ efflux in the low-K⁺ plants. In support of this idea is the data in Fig. 6, in which vanadate and cyanide, both known to rapidly depolarize the plasma membrane (Lew 1991), enhanced the efflux of 42K⁺, while treatment with bicarbonate, which hyperpolarizes the membrane (Poole 1969), reduced 42K⁺ efflux. These effects were most pronounced in low-K⁺ conditions, but were also seen in the mild stimulation of efflux by vanadate and cyanide in high-K⁺ conditions. The state of membrane electrical polarization as an explanation for changes in K⁺ efflux was also the explanation put forward by Nocito et al. (2002), who showed that the cations Rb⁺, Cs⁺, and NH₄⁺ all penetrated into cells of excised maize roots, causing depolarization and inducing K⁺ efflux, while Li⁺ and Na⁺ neither penetrated nor depolarized cells, nor induced K⁺ efflux. This attractive explanation, however, may be incomplete, as elevated nitrate in the present study reduced 42K⁺ efflux in low-K⁺ plants, despite the fact that nitrate application also results in transient membrane depolarization, because of its symport mechanism with an excess of H⁺ ions (Glass et al. 1992).
The stimulation of K⁺ efflux by high NH₄⁺, and its partial stimulation by NH₄NO₃, were mirrored in the repression of K⁺ influx by these same N sources (Fig. 5). Thus, the homeostasis of K⁺ in barley plants, in terms of both its acquisition and loss, appears to be optimized by NO₃⁻ provision and compromised, in two important ways, by the presence of NH₄⁺, which greatly stimulates efflux of K⁺ and inhibits its influx. This pattern is consistent with the sensitivity of plant species such as barley to NH₄⁺ toxicity (Britto and Kronzucker 2002). Indeed, the potency of NH₄⁺ to accelerate K⁺ efflux is greater than that of Na⁺, which, while being an important cause of stress in barley, had no effect on K⁺ efflux when applied at the same concentration as NH₄⁺ (10 mM). By contrast, the threshold for a stimulatory effect of Na⁺ was >25 mM. These differential effects warrant further investigation, particularly with regard to their agronomic consequences.

We have demonstrated here that radiotracer analysis can reveal subtle and complex aspects of K⁺ transport in intact plants, and may thus help refine the search for molecular mechanisms underlying this process. The study provides a framework of characteristics, including pharmacological and nutritional profiles, by which discoveries made at other levels of organization (e.g. molecular) might be gauged. In addition, our study provides new physiological evidence for the classic two-system model of K⁺ acquisition, and enables a grasp of the elusive efflux component of this acquisition process. Lastly, we have shown that, in addition to the pronounced effects that nitrogen source has on the influx of K⁺, K⁺ efflux plays an important role in cellular and whole-root K⁺ utilization efficiency under changing nitrogen sources.
3.2 K⁺ Efflux and Retention in Response to NaCl Stress do not Predict Salt Tolerance in Contrasting Genotypes of Rice (Oryza sativa L.)

Preface


HJ Kronzucker designed the study, with input from DT Britto and D Coskun. D Coskun performed the experiments, with input from Y-K Jean, I Kabir, I Tolay, and AA Torun. D Coskun analyzed the data, with input from DT Britto and HJ Kronzucker. D Coskun wrote the manuscript, with input and editing from DT Britto and HJ Kronzucker.

3.2.1 Abstract

Sudden elevations in external sodium chloride (NaCl) accelerate potassium (K⁺) efflux across the plasma membrane of plant root cells. It has been proposed that the extent of this acceleration can predict salt tolerance among contrasting cultivars. However, this proposal has not been considered in the context of plant nutritional history, nor has it been explored in rice (Oryza sativa L.), which stands among the world’s most important and salt-sensitive crop species. Using efflux analysis with ⁴²K, coupled with growth and tissue K⁺ analyses, we examined the short- and long-term effects of NaCl exposure to plant performance within a nutritional matrix that significantly altered tissue-K⁺ set points in three rice cultivars that differ in salt tolerance: IR29 (sensitive), IR72 (moderate), and Pokkali (tolerant). We show that total short-term K⁺ release from roots in response to NaCl stress is small (no more than 26% over 45 min) in rice. Despite strong varietal differences, the extent of efflux is shown to be a poor predictor of plant performance on long-term NaCl stress. In fact, no measure of K⁺ status was found to correlate with plant performance among cultivars either in the presence or absence of NaCl stress. By contrast, shoot Na⁺ accumulation showed the strongest correlation (a negative one) with biomass,
under long-term salinity. Pharmacological evidence suggests that NaCl-induced K⁺ efflux is a result of membrane disintegration, possibly as result of osmotic shock, and not due to ion-channel mediation. Taken together, we conclude that, in rice, K⁺ status (including efflux) is a poor predictor of salt tolerance and overall plant performance and, instead, shoot Na⁺ accumulation is the key factor in performance decline on NaCl stress.

3.2.2 Introduction

Soil salinity, predominantly in the form of NaCl, is a major agricultural issue, particularly in irrigated areas (Flowers 1999; Zhu 2001), where as much as one third of the world’s food production takes place and nearly half of the land is afflicted (Munns 2002; and references therein). In plants, one of the major consequences of salinity stress is a disruption in cellular and whole-plant K⁺ homeostasis (Rains and Epstein 1967; Maathuis and Amtmann 1999; Shabala and Cuin 2008; Kronzucker and Britto 2011). Potassium is critical to the proper functioning of plant cells for reasons that include charge balancing in the cytoplasm, enzyme activation, and the maintenance of cell turgor (Britto and Kronzucker 2008; Szczerba et al. 2009). Importantly, Na⁺ has been shown to disturb the transport processes of K⁺ across the plasma membrane, specifically in root epidermal and cortical cells where Na⁺ is first encountered, by inhibiting the primary uptake of K⁺ as well as stimulating its cellular release (Epstein et al. 1963; Kochian et al. 1985; Kronzucker et al. 2006; Shabala et al. 2006; Britto et al. 2010).

The phenomenon of NaCl-stimulated K⁺ efflux in roots has been of much recent interest, and some controversy exists regarding its underlying mechanism. Some reports have described the effect as predominantly a channel-mediated phenomenon, where it is postulated that membrane depolarization due to Na⁺ entry (possibly via non-selective cation channels, NSCCs) results in the opening of voltage-gated, outward-rectifying K⁺ channels (Shabala et al. 2006). An alternative explanation is that high amounts of NaCl compromises the integrity of the plasma membrane, due to ionic and osmotic effects, resulting in release of cellular contents, including K⁺ (Nassery 1979; Cramer et al. 1985; Britto et al. 2010). Understanding this phenomenon would provide important insight into uncovering the elusive nature of salt toxicity (Zhang et al. 2010; Kronzucker and Britto 2011), and would allow for critical assessment of the relevance of stimulated K⁺ efflux to other aspects of salt stress, such as the inhibition of primary K⁺ uptake,
cytosolic K⁺:Na⁺ ratios, primary Na⁺ uptake, and shoot Na⁺ accumulation (Maathuis and Amtmann 1999; Munns and Tester 2008; Britto et al. 2010; Kronzucker and Britto 2011; Schulze et al. 2012).

The development of salt-tolerant genotypes to meet increasing global food demands relies on effective and efficient screening methods for salt tolerance among crops (Yeo et al. 1990; Chen et al. 2005; Cuin et al. 2008; Faiyue et al. 2012). Recently, it has been proposed that assaying NaCl-stimulated K⁺ efflux in seedling roots can be one such method, as negative correlations in barley and wheat were found between the magnitude of K⁺ efflux and physiological measures/yield data in mature plants used to identify salt tolerance (Chen et al. 2005, 2007; Cuin et al. 2008). This proposal, however, has not been explored in the chief crop species, rice (Oryza sativa L.), which ranks among the most salt-sensitive crops (Yeo et al. 1990; Shannon et al. 1998; Lee et al. 2003; Ismail et al. 2007; Munns and Tester 2008). Furthermore, it has not been considered in the context of the nutritional conditions under which the plants have been reared. Studies on the effects of nitrogen (N) source (i.e., ammonium (NH₄⁺) vs. nitrate (NO₃⁻)) have reported greater sensitivity of crops to salinity when NH₄⁺ was the sole nitrogen form supplied (Lewis et al. 1989; Speer et al. 1994; Speer and Kaiser 1994; Frechilla et al. 2001). By contrast, others have shown salinity effects to be independent of N source (Bourgeais-Chaillou et al. 1992), or have reported greater sensitivity when NO₃⁻ was the sole N source (Botella et al. 1997). Moreover, it has been shown that K⁺ fluxes and cellular compartmentation can depend significantly on external N source and strength (Kronzucker et al. 2003; Coskun et al. 2010). Lastly, the application of exogenous K⁺ to alleviate plants from salinity stress is well documented (Lopez and Satti 1996; Grattan and Grieve 1999; Achilea 2002; Cakmak 2005). Thus, it is conceivable that the extent of NaCl-stimulated K⁺ efflux can differ significantly depending on growth history, particularly with respect to K⁺ and N nutrition, and should be critically considered before broader conclusions are drawn regarding the utility of such a screening tool.

In the present study, we tested the hypothesis that the extent of K⁺ efflux upon short-term exposure to NaCl can predict plant performance on long-term NaCl stress in three cultivars of rice that differ in salt sensitivity: IR29 (sensitive), IR72 (moderate), and Pokkali (tolerant). Plants were grown under eight nutritional regimes varying in N source (NH₄⁺ vs. NO₃⁻), N strength (0.1 vs. 10 mM), and K⁺ strength (0.1 vs. 1.5 mM), to investigate the effects of these two key
macronutrients to K\(^+\) status and growth, in relation to performance on short- and long-term NaCl stress. Responses to short-term NaCl stress that were considered include: (1) peak NaCl-stimulated K\(^+\) efflux, (2) cytosolic K\(^+\) release, and (3) total root K\(^+\) loss. Measures of long-term NaCl stress include: (1) survival, (2) biomass, (3) tissue K\(^+\) content, and (4) tissue Na\(^+\) content. We show that, surprisingly, no measure of K\(^+\) fluxes or accumulation could predict plant performance in the presence or absence of NaCl stress, and that instead, shoot Na\(^+\) content was the best indicator of performance on high salinity, albeit after the fact.

### 3.2.3 Results

Fig. 7 shows the release kinetics of \(^{42}\)K\(^+\) from roots of intact, pre-labeled, rice seedlings, and their response to sudden application of 160 mM NaCl (at \(t = 15.5\) min, see arrow), in three cultivars that differ in salt tolerance: IR29 (sensitive), IR72 (moderate), and Pokkali (tolerant). Seedlings were grown and measured under eight nutritional conditions that varied in N source (NH\(_4^+\) or NO\(_3^-\)), N strength (0.1 or 10 mM, referred to as ‘low’ and ‘high’, respectively), and K\(^+\) strength (0.1 or 1.5 mM, also referred to as ‘low’ and high’), which had considerable effects on plant biomass and tissue K\(^+\) content (Tables 2, 3, 4, 5, also see below).

As was previously shown in barley (Britto et al. 2010), sudden exposure of roots to 160 mM NaCl caused an immediate stimulation of \(^{42}\)K\(^+\) efflux in rice seedlings. This response was observed in all cultivars, regardless of growth condition (Fig. 7). We should note, however, that this response was not observed at lower [NaCl] (\(i.e.\) 25–75 mM; Fig. S1), although 50 mM NaCl was effective at suppressing growth in all three cultivars (see below). Salt-tolerant Pokkali displayed lower NaCl-stimulated K\(^+\) efflux, relative to the other cultivars, in terms of both peak efflux and an integration of all \(^{42}\)K\(^+\) released during elution (Table 6), under all growth conditions except for low K\(^+\), high NH\(_4^+\) (Fig. 7D). By contrast, although IR72 displayed intermediate salt sensitivity (as measured by survival, biomass decline, and shoot Na\(^+\) content; Tables 2, 3, 4, 5), this was not generally reflected in the extent of NaCl-stimulated K\(^+\) efflux. In fact, only under low nitrate conditions did efflux in IR72 fall between that of IR29 and Pokkali (Fig. 7B and F).
Figure 7. Nutritional and cultivar comparisons of NaCl-stimulated K+ efflux. Cultivar differences in $^{42}\text{K}^+$ efflux from roots of intact rice (*Oryza sativa* L. cvs. ‘IR29’, ‘IR72’, and ‘Pokkali’) in response to sudden provision (at $t = 15.5$ min, see arrow) of 160 mM NaCl. Seedlings were grown and tested in a full-nutrient medium supplemented with either 0.1 (A – D) or 1.5 mM K+ (E – H), and one of four N regimes: 0.1 mM NH$_4^+$ (A, E), 0.1 mM NO$_3^-$ (B, F), 10 mM NH$_4^+$ (C, G), and 10 mM NO$_3^-$ (D, H). Error bars indicate ± SEM.
Fig. 8 illustrates the sensitivity of NaCl-stimulated K\textsuperscript{+} efflux in IR72 to selected ion channel inhibitors. Under the conditions tested, NaCl-stimulated K\textsuperscript{+} efflux showed no sensitivity to Cs\textsuperscript{+}, a potent inhibitor of K\textsuperscript{+} channels, including outward-rectifying K\textsuperscript{+} channels (Roberts and Tester 1995; Coskun et al. 2010; see also Subchapter 3.1). By contrast, NaCl-stimulated K\textsuperscript{+} efflux displayed significant sensitivity to added Ca\textsuperscript{2+}, which is known to both inhibit NSCCs (Roberts and Tester 1997; Tyerman et al. 1997; Davenport and Tester 2000) and stabilize membranes (Cramer et al. 1985; Rengel 1992; Kinraide 1999). This was particularly noticeable under low-K\textsuperscript{+} conditions (Fig. 8A).

Total K\textsuperscript{+} content of roots before and after short-term NaCl stress (45-min exposure to 160 mM NaCl) showed relatively little decline (Fig. 9). No more than 20 µmol K\textsuperscript{+} g\textsuperscript{-1} FW were lost (see IR72 at high K\textsuperscript{+}, high NO\textsubscript{3}\textsuperscript{-}; Table 6), which amounted to a maximal decline of 26% compared to control (~78 µmol g\textsuperscript{-1}; Fig. 9, Table 5). These losses were considerably smaller than the differences in root K\textsuperscript{+} content among cultivars in the absence of NaCl stress, where amounts ranged between 24 µmol g\textsuperscript{-1} FW (at low K\textsuperscript{+}, high NH\textsubscript{4}\textsuperscript{+}) and 112 µmol g\textsuperscript{-1} FW (high K\textsuperscript{+}, low NH\textsubscript{4}\textsuperscript{+}) (Fig. 9; Table 2 and Table 4, respectively). In the presence of long-term NaCl stress, root K\textsuperscript{+} content ranged from 18 to 52 µmol g\textsuperscript{-1} FW, depending on growth history, amounting to a maximal decline of 70% compared to control (see IR72 at low K\textsuperscript{+}, low NH\textsubscript{4}\textsuperscript{+}; Table 2).

No measure of K\textsuperscript{+} status could predict plant performance either in the presence or absence of NaCl stress. When combining data from all cultivars and conditions, neither root nor shoot K\textsuperscript{+} content showed a correlation with FW in the absence (Fig. 10A) or presence (Fig. 10B) of long-term NaCl stress. Moreover, no general relationship was found between plant performance under long-term NaCl stress and the magnitudes of NaCl-stimulated peak K\textsuperscript{+} efflux, integrated K\textsuperscript{+} efflux or root K\textsuperscript{+} decline (Table S1). In fact, in only one scenario could a strong negative correlation (R\textsuperscript{2} > 0.94) be found between peak K\textsuperscript{+} efflux and tissue biomass under long-term NaCl stress (Fig. 11B, inset: roots at high K\textsuperscript{+}). No correlations were found under low K\textsuperscript{+} conditions for Pokkali (Fig. 11B), and surprisingly, significant positive correlations were found for both shoot and root tissue for IR72 at both K\textsuperscript{+} levels (Fig. 11A).

In contrast to these findings with K\textsuperscript{+}, shoot Na\textsuperscript{+} content showed a strong negative correlation (R\textsuperscript{2} = 0.77) with shoot biomass under long-term NaCl stress (Fig. 10C). This was not the case for root tissue (Fig. 10C, inset).
Figure 8. Inhibitor effects of NaCl-stimulated K⁺ efflux. The effect of co-application of 10 mM CsCl or CaCl₂ with sudden provision (at $t = 15.5$ min, see arrow) of 160 mM NaCl on the response of $^{42}$K⁺ efflux from roots of intact rice (Oryza sativa L.) in the cultivar IR72. External N source was supplied as 10 mM NH₄⁺ and K⁺ at either 0.1 (A) or 1.5 mM (B). Error bars indicate ± SEM.
A

0.1 mM K⁺

![Graph showing Root K⁺ content (µmol g⁻¹ FW) for different treatments and genotypes.](image)

B

1.5 mM K⁺

![Graph showing Root K⁺ content (µmol g⁻¹ FW) for different treatments and genotypes.](image)
Figure 9. Root K⁺ content and short-term NaCl stress. Root K⁺ content, before and after short-term (45 min) exposure to 160 mM NaCl, in three cultivars of rice (Oryza sativa L., cvs. ‘IR29’, ‘IR72’, and ‘Pokkali’). Plants were grown and tested in a full nutrient medium supplemented with either 0.1 (A) or 1.5 mM K⁺ (B), and one of four N regimes: 0.1 mM NH₄⁺, 0.1 mM NO₃⁻, 10 mM NH₄⁺, and 10 mM NO₃⁻. Asterisks denote different levels of significance between control and treatment pairs (ns: not significant, *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001; Student’s t-test). Error bars indicate ± SEM.
Figure 10. Tissue $K^+$/Na$^+$ content and biomass. Correlation analyses between shoot $K^+$ content and fresh weight, in the absence (A) or presence (B) of long-term NaCl stress, and shoot Na$^+$ content and fresh weight in the presence of long-term NaCl stress (C). Data was accumulated from three cultivars of rice (*Oryza sativa* L.) grown under varying nutritional conditions. Inset: respective correlation analyses between ion content and fresh weight for root tissues.
Figure 11. NaCl-stimulated K+ efflux and biomass. Correlation analyses between NaCl-stimulated peak K+ efflux from roots and total biomass (or shoot and root biomass, separately; insets) on long-term NaCl stress in (A) IR72 and (B) Pokkali under low and high K+ growth conditions. Axes labels for insets as in main figure. Error bars indicate ± SEM.
Table 2. Long-term NaCl exposure and tissue biomass and content (low K⁺, NH₄⁺ conditions).

<table>
<thead>
<tr>
<th></th>
<th>0.1 mM NH₄⁺</th>
<th>0.1 mM K⁺</th>
<th>10 mM NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR29</td>
<td>IR72</td>
<td>Pokkali</td>
</tr>
<tr>
<td>Root DW:FW</td>
<td>-Na⁺</td>
<td>+Na⁺</td>
<td>0.703 ± 0.061</td>
</tr>
<tr>
<td>Shoot FW (g)</td>
<td>-0.393 ± 0.049</td>
<td>0.328 ± 0.023</td>
<td>0.988 ± 0.051</td>
</tr>
<tr>
<td>Root FW (g)</td>
<td>0.577 ± 0.035</td>
<td>0.360 ± 0.030</td>
<td>0.360 ± 0.030</td>
</tr>
<tr>
<td>Total FW (g)</td>
<td>-1.096 ± 0.109</td>
<td>-1.342 ± 0.096</td>
<td>-1.565 ± 0.083</td>
</tr>
<tr>
<td>Total FW decline (g)</td>
<td>-0.768 ± 0.109</td>
<td>0.459 ± 0.123</td>
<td>-0.768 ± 0.109</td>
</tr>
<tr>
<td>Total FW decline (%)</td>
<td>57.23</td>
<td>29.33</td>
<td>-</td>
</tr>
<tr>
<td>Shoot DW:FW ratio</td>
<td>0.190 ± 0.001</td>
<td>0.182 ± 0.001</td>
<td>0.233 ± 0.006</td>
</tr>
<tr>
<td>Root DW:FW ratio</td>
<td>0.082 ± 0.004</td>
<td>0.083 ± 0.004</td>
<td>0.066 ± 0.004</td>
</tr>
<tr>
<td>SKC⁴ (µmol g⁻¹ FW)</td>
<td>169.9 ± 2.0</td>
<td>185.7 ± 1.5</td>
<td>188.4 ± 10.0</td>
</tr>
<tr>
<td>RKC⁵ (µmol g⁻¹ FW)</td>
<td>73.4 ± 0.3</td>
<td>76.4 ± 1.4</td>
<td>23.2 ± 0.5</td>
</tr>
<tr>
<td>RKC decline (µmol g⁻¹ FW)</td>
<td>-</td>
<td>53.3 ± 2.3***</td>
<td>40.5 ± 2.2***</td>
</tr>
<tr>
<td>RKC decline (%) Ctrl</td>
<td>-</td>
<td>69.63</td>
<td>58.53</td>
</tr>
<tr>
<td>SNC⁶ (µmol g⁻¹ FW)</td>
<td>12.1 ± 0.9</td>
<td>15.0 ± 0.9</td>
<td>574.6 ± 63.5</td>
</tr>
<tr>
<td>RNC⁷ (µmol g⁻¹ FW)</td>
<td>15.5 ± 1.3</td>
<td>14.4 ± 0.9</td>
<td>167.4 ± 20.3</td>
</tr>
</tbody>
</table>

Steady-state biomass and tissue content values of three rice (Oryza sativa L.) cultivars grown under low K⁺ and NH₄⁺ conditions, +/- 50 mM NaCl. Dashes indicate instances of mortality. Asterisks denote different levels of significance between control and treatment pairs (ns: not significant, *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***: P < 0.001; Student’s t-test). ¹ Fresh weight; ² Control; ³ Dry weight; ⁴ Shoot K⁺ content; ⁵ Root K⁺ content; ⁶ Shoot Na⁺ content; ⁷ Root Na⁺ content.
Table 3. Long-term NaCl exposure and tissue biomass and content (low K⁺, NO₃⁻ conditions).

<table>
<thead>
<tr>
<th></th>
<th>0.1 mM NO₃⁻</th>
<th></th>
<th>0.1 mM K⁺</th>
<th></th>
<th>10 mM NO₃⁻</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR29</td>
<td>IR72</td>
<td>Pokkali</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokkali</td>
</tr>
<tr>
<td>Root DW:FW ratio</td>
<td>-Na⁺</td>
<td>+Na⁺</td>
<td>-Na⁺</td>
<td>+Na⁺</td>
<td>-Na⁺</td>
<td>+Na⁺</td>
</tr>
<tr>
<td>Root FW (g)</td>
<td>0.866 ± 0.036</td>
<td>-</td>
<td>0.864 ± 0.044</td>
<td>0.214 ± 0.031</td>
<td>1.363 ± 0.126</td>
<td>0.582 ± 0.026</td>
</tr>
<tr>
<td>Shoot FW¹ (g)</td>
<td>0.610 ± 0.034</td>
<td>-</td>
<td>0.754 ± 0.025</td>
<td>0.168 ± 0.027</td>
<td>1.047 ± 0.055</td>
<td>0.362 ± 0.014</td>
</tr>
<tr>
<td>Total FW (g)</td>
<td>1.476 ± 0.062</td>
<td>-</td>
<td>1.618 ± 0.068</td>
<td>0.382 ± 0.058</td>
<td>2.410 ± 0.176</td>
<td>0.944 ± 0.039</td>
</tr>
<tr>
<td>Total FW decline (g)</td>
<td>-</td>
<td>1.236 ± 0.090</td>
<td>1.466 ± 0.181</td>
<td>0.687 ± 0.164</td>
<td>0.565 ± 0.091</td>
<td>1.147 ± 0.114</td>
</tr>
<tr>
<td>Total FW decline (% Ctrl²)</td>
<td>-</td>
<td>76.39</td>
<td>60.83</td>
<td>53.63</td>
<td>43.06</td>
<td>48.36</td>
</tr>
<tr>
<td>SKC (µmol g⁻¹ FW)</td>
<td>199.8 ± 1.4</td>
<td>-</td>
<td>177.9 ± 1.7</td>
<td>188.1 ± 25.1</td>
<td>179.1 ± 3.4</td>
<td>120.0 ± 3.2</td>
</tr>
<tr>
<td>RKC (µmol g⁻¹ FW)</td>
<td>92.7 ± 1.2</td>
<td>-</td>
<td>85.7 ± 1.6</td>
<td>33.4 ± 3.5</td>
<td>81.3 ± 4.7</td>
<td>27.6 ± 1.2</td>
</tr>
<tr>
<td>RKC decline (µmol g⁻¹ FW)</td>
<td>-</td>
<td>52.3 ± 3.4</td>
<td>53.7 ± 4.8</td>
<td>32.1 ± 5.1</td>
<td>16.5 ± 4.8</td>
<td>23.6 ± 1.7</td>
</tr>
<tr>
<td>RKC decline (% Ctrl)</td>
<td>-</td>
<td>61.03</td>
<td>66.05</td>
<td>58.15</td>
<td>34.88</td>
<td>47.01</td>
</tr>
<tr>
<td>SNC (µmol g⁻¹ FW)</td>
<td>27.1 ± 5.4</td>
<td>-</td>
<td>16.1 ± 1.3</td>
<td>583.2 ± 138.8</td>
<td>14.5 ± 1.4</td>
<td>297.5 ± 29.3</td>
</tr>
<tr>
<td>RNC (µmol g⁻¹ FW)</td>
<td>16.3 ± 1.2</td>
<td>-</td>
<td>14.4 ± 0.5</td>
<td>117.6 ± 7.6</td>
<td>16.2 ± 0.7</td>
<td>107.6 ± 2.2</td>
</tr>
</tbody>
</table>

Steady-state biomass and tissue content values of three rice (*Oryza sativa L.*) cultivars grown under low K⁺ and NO₃⁻ conditions, +/- 50 mM NaCl. Dashes indicate instances of mortality. Asterisks denote different levels of significance between control and treatment pairs (ns: not significant, *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***: P < 0.001; Student’s t-test). ¹ Fresh weight; ² Control; ³ Dry weight; ⁴ Shoot K⁺ content; ⁵ Root K⁺ content; ⁶ Shoot Na⁺ content; ⁷ Root Na⁺ content.
Table 4. Long-term NaCl exposure and tissue biomass and content (high K⁺, NH₄⁺ conditions).

<table>
<thead>
<tr>
<th></th>
<th>1.5 mM K⁺</th>
<th>0.1 mM NH₄⁺</th>
<th>10 mM NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR29</td>
<td>IR72</td>
<td>Pokkali</td>
</tr>
<tr>
<td>Shoot FW (g)</td>
<td>-Na⁺</td>
<td>+Na⁺</td>
<td>-Na⁺</td>
</tr>
<tr>
<td></td>
<td>0.485 ± 0.076</td>
<td>-</td>
<td>0.937 ± 0.104</td>
</tr>
<tr>
<td>Root FW (g)</td>
<td>0.248 ± 0.048</td>
<td>-</td>
<td>0.536 ± 0.059</td>
</tr>
<tr>
<td>Total FW (g)</td>
<td>0.734 ± 0.124</td>
<td>-</td>
<td>1.473 ± 0.161</td>
</tr>
<tr>
<td>Total FW decline (g)</td>
<td>-</td>
<td>1.120 ± 0.178***</td>
<td>0.287 ± 0.217***</td>
</tr>
<tr>
<td>Total FW decline (%) Ctrl²</td>
<td>-</td>
<td>76.04</td>
<td>16.13</td>
</tr>
<tr>
<td>Shoot DW³:FW ratio</td>
<td>0.184 ± 0.001</td>
<td>-</td>
<td>0.165 ± 0.002</td>
</tr>
<tr>
<td>Root DW:FW ratio</td>
<td>0.066 ± 0.003</td>
<td>-</td>
<td>0.073 ± 0.001</td>
</tr>
<tr>
<td>SKC⁴ (µmol g⁻¹ FW)</td>
<td>202.3 ± 5.1</td>
<td>-</td>
<td>194.4 ± 3.0</td>
</tr>
<tr>
<td>RKC⁵ (µmol g⁻¹ FW)</td>
<td>105.4 ± 1.8</td>
<td>-</td>
<td>105.0 ± 3.5</td>
</tr>
<tr>
<td>RKC decline (µmol g⁻¹ FW)</td>
<td>-</td>
<td>63.5 ± 6.2***</td>
<td>60.7 ± 4.0***</td>
</tr>
<tr>
<td>RKC decline (%) Ctrl</td>
<td>-</td>
<td>60.38</td>
<td>54.10</td>
</tr>
<tr>
<td>SNC⁶ (µmol g⁻¹ FW)</td>
<td>17.7 ± 3.6</td>
<td>-</td>
<td>13.7 ± 1.6</td>
</tr>
<tr>
<td>RNC⁷ (µmol g⁻¹ FW)</td>
<td>11.8 ± 2.1</td>
<td>-</td>
<td>11.2 ± 1.0</td>
</tr>
</tbody>
</table>

Steady-state biomass and tissue content values of three rice (Oryza sativa L.) cultivars grown under high K⁺ and NH₄⁺ conditions, +/- 50 mM NaCl. Dashes indicate instances of mortality. Asterisks denote different levels of significance between control and treatment pairs (ns: not significant, *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***: P < 0.001; Student’s t-test). ¹ Fresh weight; ² Control; ³ Dry weight; ⁴ Shoot K⁺ content; ⁵ Root K⁺ content; ⁶ Shoot Na⁺ content; ⁷ Root Na⁺ content.
Table 5. Long-term NaCl exposure and tissue biomass and content (high K\(^+\), NO\(_3^-\) conditions).

<table>
<thead>
<tr>
<th></th>
<th>1.5 mM K(^+)</th>
<th>0.1 mM NO(_3^-)</th>
<th>Pokkali</th>
<th>10 mM NO(_3^-)</th>
<th>Pokkali</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR29</td>
<td>IR72</td>
<td></td>
<td>IR29</td>
<td>IR72</td>
</tr>
<tr>
<td></td>
<td>-Na(^+)</td>
<td>+Na(^+)</td>
<td>-Na(^+)</td>
<td>+Na(^+)</td>
<td>-Na(^+)</td>
</tr>
<tr>
<td>Shoot FW(^1) (g)</td>
<td>0.687 ± 0.095</td>
<td>0.798 ± 0.058</td>
<td>1.425 ± 0.113</td>
<td>1.029 ± 0.057</td>
<td>0.814 ± 0.065</td>
</tr>
<tr>
<td></td>
<td>0.069</td>
<td>0.044</td>
<td>0.928 ± 0.098</td>
<td>0.572 ± 0.023</td>
<td>0.348 ± 0.030</td>
</tr>
<tr>
<td>Root FW (g)</td>
<td>0.462 ± 0.069</td>
<td>0.487 ± 0.044</td>
<td>0.828 ± 0.098</td>
<td>0.572 ± 0.023</td>
<td>0.348 ± 0.030</td>
</tr>
<tr>
<td>Total FW (g)</td>
<td>1.149 ± 0.135</td>
<td>1.285 ± 0.099</td>
<td>2.352 ± 0.201</td>
<td>1.601 ± 0.076</td>
<td>1.162 ± 0.094</td>
</tr>
<tr>
<td>Total FW decline (g)</td>
<td>-</td>
<td>-</td>
<td>0.751 ± 0.215*</td>
<td>-</td>
<td>1.048 ± 0.131**</td>
</tr>
<tr>
<td>Total FW decline (% Ctrl(^2))</td>
<td>-</td>
<td>-</td>
<td>31.93</td>
<td>-</td>
<td>72.33</td>
</tr>
<tr>
<td>Shoot DW(^3):FW ratio</td>
<td>0.166 ± 0.003</td>
<td>0.170 ± 0.003</td>
<td>0.158 ± 0.001</td>
<td>0.158 ± 0.001</td>
<td>0.170 ± 0.002</td>
</tr>
<tr>
<td>Root DW:FW ratio</td>
<td>0.070 ± 0.002</td>
<td>0.074 ± 0.002</td>
<td>0.091 ± 0.001</td>
<td>0.072 ± 0.001</td>
<td>0.070 ± 0.001</td>
</tr>
<tr>
<td>SKC(^4) (µmol g(^{-1}) FW)</td>
<td>230.3 ± 6.5</td>
<td>202.7 ± 4.6</td>
<td>202.1 ± 2.9</td>
<td>157.9 ± 4.7</td>
<td>184.8 ± 2.8</td>
</tr>
<tr>
<td>RKC(^5) (µmol g(^{-1}) FW)</td>
<td>100.9 ± 4.0</td>
<td>99.6 ± 3.2</td>
<td>99.5 ± 4.2</td>
<td>46.7 ± 1.4</td>
<td>83.3 ± 2.6</td>
</tr>
<tr>
<td>RKC decline (µmol g(^{-1}) FW)</td>
<td>-</td>
<td>-</td>
<td>52.8 ± 6.8***</td>
<td>-</td>
<td>32.3 ± 4.4***</td>
</tr>
<tr>
<td>RKC decline (% Ctrl)</td>
<td>-</td>
<td>-</td>
<td>53.07</td>
<td>-</td>
<td>45.55</td>
</tr>
<tr>
<td>SNC(^6) (µmol g(^{-1}) FW)</td>
<td>20.2 ± 5.6</td>
<td>12.3 ± 1.1</td>
<td>14.7 ± 2.1</td>
<td>143.8 ± 7.4</td>
<td>19.3 ± 1.3</td>
</tr>
<tr>
<td>RNC(^7) (µmol g(^{-1}) FW)</td>
<td>8.6 ± 0.4</td>
<td>5.9 ± 0.4</td>
<td>26.5 ± 0.6</td>
<td>82.0 ± 3.3</td>
<td>12.7 ± 1.6</td>
</tr>
</tbody>
</table>

Steady-state biomass and tissue content values of three rice (*Oryza sativa* L.) cultivars grown under high K\(^+\) and NO\(_3^-\) conditions, +/- 50 mM NaCl. Dashes indicate instances of mortality. Asterisks denote different levels of significance between control and treatment pairs (ns: not significant, *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***: P < 0.001; Student’s t-test). 1 Fresh weight; 2 Control; 3 Dry weight; 4 Shoot K\(^+\) content; 5 Root K\(^+\) content; 6 Shoot Na\(^+\) content; 7 Root Na\(^+\) content.
Table 6. Short-term NaCl exposure and K⁺ efflux and retention.

<table>
<thead>
<tr>
<th></th>
<th>0.1 mM K⁺</th>
<th></th>
<th>0.1 mM K⁺</th>
<th></th>
<th>0.1 mM NO₃</th>
<th></th>
<th>0.1 mM NO₃</th>
<th></th>
<th>1.5 mM K⁺</th>
<th></th>
<th>1.5 mM K⁺</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mM NH₄⁺</td>
<td>10 mM NH₄⁺</td>
<td>0.1 mM NO₃</td>
<td>10 mM NO₃</td>
<td>0.1 mM NH₄⁺</td>
<td>10 mM NH₄⁺</td>
<td>0.1 mM NO₃</td>
<td>10 mM NO₃</td>
<td>0.1 mM NH₄⁺</td>
<td>10 mM NH₄⁺</td>
<td>0.1 mM NO₃</td>
<td>10 mM NO₃</td>
</tr>
<tr>
<td>peak efflux (µmol g⁻¹ h⁻¹)</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
</tr>
<tr>
<td>K⁺ efflux</td>
<td>0.026 ± 0.004</td>
<td>0.037 ± 0.004</td>
<td>0.018 ± 0.001</td>
<td>0.028 ± 0.003</td>
<td>0.032 ± 0.005</td>
<td>0.034 ± 0.002</td>
<td>0.017 ± 0.001</td>
<td>0.010 ± 0.002</td>
<td>0.049 ± 0.014</td>
<td>0.073 ± 0.011</td>
<td>0.025 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>K⁺ cytosolic loss (µmol g⁻¹)</td>
<td>0.677 ± 0.096</td>
<td>0.886 ± 0.084</td>
<td>0.435 ± 0.048</td>
<td>0.519 ± 0.049</td>
<td>0.660 ± 0.089</td>
<td>0.789 ± 0.117</td>
<td>0.898 ± 0.083</td>
<td>0.430 ± 0.009</td>
<td>0.227 ± 0.041</td>
<td>0.441 ± 0.081</td>
<td>0.667 ± 0.075</td>
<td>0.419 ± 0.019</td>
</tr>
<tr>
<td>K⁺ tissue loss (µmol g⁻¹)</td>
<td>1.224 ± 0.992</td>
<td>3.371 ± 1.769</td>
<td>-8.911 ± 3.859</td>
<td>n.d.</td>
<td>7.100 ± 1.508</td>
<td>7.547 ± 0.941</td>
<td>10.690 ± 3.038</td>
<td>7.185 ± 2.368</td>
<td>7.129 ± 4.270</td>
<td>3.063 ± 3.627</td>
<td>9.165 ± 1.704</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
<td>IR29</td>
<td>IR72</td>
<td>Pokk</td>
</tr>
<tr>
<td>peak efflux (µmol g⁻¹ h⁻¹)</td>
<td>0.042 ± 0.007</td>
<td>0.046 ± 0.006</td>
<td>0.021 ± 0.004</td>
<td>0.052 ± 0.003</td>
<td>0.069 ± 0.012</td>
<td>0.039 ± 0.006</td>
<td>0.035 ± 0.008</td>
<td>0.020 ± 0.001</td>
<td>0.016 ± 0.001</td>
<td>0.077 ± 0.013</td>
<td>0.076 ± 0.011</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>K⁺ cytosolic loss (µmol g⁻¹)</td>
<td>0.980 ± 0.171</td>
<td>1.051 ± 0.126</td>
<td>0.452 ± 0.096</td>
<td>1.102 ± 0.079</td>
<td>1.426 ± 0.201</td>
<td>0.950 ± 0.135</td>
<td>0.934 ± 0.207</td>
<td>0.486 ± 0.027</td>
<td>0.324 ± 0.015</td>
<td>0.571 ± 0.045</td>
<td>0.678 ± 0.083</td>
<td>0.406 ± 0.057</td>
</tr>
</tbody>
</table>

Effect of sudden application of 160 mM NaCl to K⁺ efflux and K⁺ content loss (from the cytosol and total tissue) from roots of three rice (Oryza sativa L.) cultivars grown under varying nutritional conditions. Letters indicate significantly different means (one-way ANOVA with Bonferroni post-test, P < 0.05) between cultivars within an N regime. Asterisks denote different levels of significance between control and treatment pairs used to determine K⁺ tissue loss (ns: not significant, *: 0.01 < P < 0.05, **: 0.001 < P < 0.01, ***: P < 0.001; Student’s t-test). ¹ cytosolic K⁺; ² total tissue K⁺; ³ not determined.
3.2.4 Discussion

The present study is the first to examine NaCl-stimulated K+ efflux in rice and to relate this phenomenon to performance on long-term NaCl stress. Consistent with studies on other plant species, e.g. barley (Chen et al. 2005; Britto et al. 2010), wheat (Cuin et al. 2008), bean (Nassery 1975), cotton (Cramer et al. 1985), Arabidopsis (Shabala et al. 2006), pea (Shabala et al. 2007), alfalfa (Smethurst et al. 2008), and sunflower (our unpublished results), we show that sudden exposure to high levels of NaCl produce a significant and sustained stimulation of K+ efflux in three cultivars of rice that differ dramatically in salt tolerance. We also show that this effect occurs regardless of nutritional history (Fig. 7), albeit to varying extents (Table 6). We should stress that this effect only occurs if NaCl concentrations are sufficiently high (e.g. 160 mM), as it was not observed in a lower range (25–75 mM; Fig. S1). By contrast, long-term exposure to 50 mM NaCl was sufficient to bring about toxicity in all cultivars, and in some cases even mortality (Tables 2, 3, 4, 5). These findings question the universal relevance of NaCl-stimulated K+ efflux to NaCl toxicity.

Recently, we investigated the mechanism underlying the efflux stimulation in barley roots, and concluded that membrane disintegrity due to osmotic and ionic effects was the cause (Britto et al. 2010), a conclusion that agreed with earlier explanations (Nassery 1979; Cramer et al. 1985), but opposed more recent explanations that attribute the effect to the gating of outwardly rectifying K+ channels by Na+-induced membrane depolarization (Shabala et al. 2006). In the present study in rice, we found that, as in barley, NaCl-stimulated K+ efflux showed no sensitivity to Cs+ (Fig. 8), an especially potent inhibitor of K+ fluxes (Król and Trebacz 2000; White and Broadley 2000; Coskun et al. 2010), which discounts the involvement of outward-rectifying K+ channels. Simultaneous application of 160 mM NaCl with 10 mM Ca2+ showed significant suppressions of K+-efflux stimulation (Fig. 8). While Ca2+ is known to inhibit some ion channels (Roberts and Tester 1997; Tyerman et al. 1997; Davenport and Tester 2000), it is also well documented that calcium is critical to the stability of membranes including under NaCl stress (Garybobo 1970; Rengel 1992; Hepler 2005), which may explain the suppression observed in the present study.

The agronomic importance of NaCl-stimulated K+ efflux has been suggested by the inverse relationship between the extent of efflux and the salt tolerance of wheat and barley cultivars, which thus may prove to be a valuable screening tool for some crops (Chen et al. 2005, 2008;
Cuin et al. 2008). In our study, the salt-tolerant cultivar, Pokkali, did show significantly lower NaCl-stimulated K\(^{+}\) efflux compared to IR29 and IR72, under all conditions but one (Fig. 7). However, the stimulation of K\(^{+}\) efflux in IR72 did not fall between that of IR29 and Pokkali under most conditions (Fig. 7), even though IR72 clearly demonstrated intermediate sensitivity to long-term NaCl stress, in terms of survival, biomass decline, and shoot Na\(^{+}\) content (Tables 2, 3, 4, 5).

Nor was there a strong negative relationship observed between peak NaCl-stimulated K\(^{+}\) efflux and plant growth on NaCl, within the three cultivars of rice examined here, under varying nutritional conditions (Fig. 11). Within cultivars, only when correlation analyses were limited to a specific K\(^{+}\) level were any relationships observed. Even then, only one correlation was strongly negative for Pokkali (roots at high K\(^{+}\); Fig. 11B inset), while all correlations were in fact strongly positive for IR72 (Fig. 11A).

Long-term NaCl stress showed no correlation between peak (or integrated) efflux, and survival, biomass decline (both absolute and relative), tissue K\(^{+}\) content and its decline (both absolute and relative), and tissue Na\(^{+}\) content and its accumulation (both absolute and relative) (Table S1). Thus, it appears that in rice, NaCl-stimulated K\(^{+}\) efflux from the root system provides no utility in screening for performance under salinity stress.

Perhaps of greater surprise was the more fundamental observation that tissue K\(^{+}\) content showed no relationship with plant biomass in even the absence of salinity stress (Fig. 10; Table S1). It has long been known that ‘luxury consumption’ of K\(^{+}\) occurs when it is not nutritionally limiting (Bartholomew and Janssen 1929; Hoagland and Martin 1933; Chapin 1980). Because plants can homeostatically maintain cytosolic [K\(^{+}\)] at 100 mM at the expense of vacuolar stores (Walker et al. 1996a), they can maintain proper functioning against a background of widely varying tissue K\(^{+}\) levels. As shown in Table 2, these levels can be extremely low, as in the case of Pokkali at low K\(^{+}\), high NH\(_{4}\)\(^{+}\), and 50 mM NaCl (18 and 40 \(\mu\)mol K\(^{+}\) g\(^{-1}\) FW in root and shoot, respectively), but can nevertheless be compatible with biomass that exceeds what is seen in other cultivars with much higher tissue K\(^{+}\) levels (e.g. IR29 and IR72 at low K\(^{+}\), high NH\(_{4}\)\(^{+}\), and without NaCl). Fig. 9 demonstrates that the loss of root K\(^{+}\) due to sudden NaCl exposure is relatively minor compared to the vast fluctuations in root K\(^{+}\) levels achieved by alterations in growth history in the absence of salt stress. Moreover, in some cases, it appears that increased K\(^{+}\)
provision can in fact be detrimental to performance on long-term NaCl exposure. Except in plants grown on high NH$_4^+$, where it is clear that enhanced K$^+$ availability is beneficial due to the alleviation of NH$_4^+$ toxicity (Balkos et al. 2010), biomass decline due to long-term NaCl exposure was actually greater on high K$^+$ in IR72 under low NH$_4^+$ and high NO$_3^-$ conditions. Furthermore, IR72 and IR29 did not survive at high K$^+$ on low and high NO$_3^-$, respectively (Table 5). Thus, it becomes apparent that, at least in rice, focus on K$^+$ status as a measure of plant performance under saline and non-saline conditions, is perhaps misguided.

By contrast, shoot Na$^+$ content was a good predictor of biomass on long-term NaCl stress ($R^2 = 0.77$; Fig. 10C). This is in good agreement with previous reports on rice that demonstrate strong negative correlations between shoot Na$^+$ content and performance (Yeo and Flowers 1983, 1982). Moreover, this was the only measure that displayed clear cultivar differences in the present work, based on salt tolerance, independent of growth history (i.e. IR29 > IR72 > Pokkali; Tables 2, 3, 4, 5). It is believed that shoot Na$^+$ accumulation in rice occurs preferentially via an apoplastic bypass pathway (Yeo et al. 1987; Garcia et al. 1997), but is lower in salt-tolerant cultivars such as Pokkali (Krishnamurthy et al. 2011). It is also believed that elevated Ca$^{2+}$ levels can reduce bypass flow of Na$^+$ into the transpiration stream (Anil et al. 2005). Indeed, under low-K$^+$, high Ca$^{2+}$ (high-NO$_3^-$) conditions, all cultivars showed the lowest shoot Na$^+$ content when grown on NaCl (Table 3). However, this was not observed at high K$^+$, which may be related to the poorer performance on salinity with high K$^+$, as mentioned above. Thus, it appears that monitoring K$^+$ nutrition (including efflux and retention) in hopes of screening for salt tolerance in rice is not a promising strategy, and that focus should remain on shoot Na$^+$ accumulation and the mechanisms by which it is brought about (Faiyue et al. 2012).
Chapter 4
K⁺ Influx in Roots of Higher Plants: Mechanisms and Connections to Abiotic Stress

4.1 Capacity and Plasticity of K⁺ Channels and High-Affinity Transporters in Roots of Barley (Hordeum vulgare L.) and Arabidopsis thaliana L.

Preface

A modified version of this subchapter was published as: Coskun D, Britto DT, Li M, Oh S, Kronzucker HJ (2013) Capacity and plasticity of K⁺ channels and high-affinity transporters in roots of barley (Hordeum vulgare L.) and Arabidopsis thaliana L. Plant Physiology 162, 496-511. Supplemental material cited in this subchapter can be found online: http://www.plantphysiol.org/content/162/1/496.

HJ Kronzucker designed the study, with input from DT Britto and D Coskun. D Coskun performed the experiments and analyzed the data, with input from M Ling and S Oh. D Coskun wrote the manuscript, with input and editing from DT Britto and HJ Kronzucker.

4.1.1 Abstract

The role of potassium (K⁺) transporters in high- and low-affinity K⁺ uptake was examined in roots of intact barley (Hordeum vulgare) and Arabidopsis (Arabidopsis thaliana) plants by use of ⁴²K radiotracing, electrophysiology, pharmacology, and mutant analysis. Comparisons were made between results from barley and five genotypes of Arabidopsis, including single and double knockout mutants for the high-affinity transporter, AtHAK5, and the Shaker-type channel, AtAKT1. In Arabidopsis, steady-state K⁺ influx at low external K⁺ concentration ([K⁺]₆ₓₐ₉ = 22.5 µM) was predominantly mediated by AtAKT1 when high-affinity transport was inhibited by ammonium, whereas in barley, by contrast, K⁺ channels could not operate below 100 µM. Withdrawal of ammonium resulted in an immediate and dramatic stimulation of K⁺ influx in barley, indicating a shift from active to passive K⁺ uptake at low [K⁺]₆ₓₐ₉ and yielding
fluxes as high as 36 µmol g\(^{-1}\) (root fresh weight) h\(^{-1}\) at 5 mM [K\(^+\)]\(_{\text{ext}}\), among the highest transporter-mediated K\(^+\) fluxes hitherto reported. This ammonium-withdrawal effect was also established in all Arabidopsis lines (the wild types, atakt1, athak5, and athak5 atakt1) at low [K\(^+\)]\(_{\text{ext}}\), revealing the concerted involvement of several transport systems. The ammonium-withdrawal effect coincided with a suppression of K\(^+\) efflux and a significant hyperpolarization of the plasma membrane in all genotypes except athak5 atakt1, could be sustained over 24 h, and resulted in increased tissue K\(^+\) accumulation. We discuss key differences and similarities in K\(^+\) acquisition between two important model systems and reveal novel aspects of K\(^+\) transport \textit{in planta}.

4.1.2 Introduction

Potassium (K\(^+\)), a major macronutrient in plants, is the most abundant intracellular cation (constituting up to 10% of plant dry weight) and is critical to such cellular functions as osmotic balance, enzyme activation, and electrical regulation (Leigh and Jones 1984; Maathuis and Sanders 1996b; Britto and Kronzucker 2008). Understanding the mechanisms of K\(^+\) acquisition in plants has long been of scientific and practical importance and is increasingly urgent in light of major ecological and agricultural problems, such as soil K\(^+\) level decline (Ashley et al. 2006; and references therein) and sodium (Na\(^+\)) and ammonium (NH\(_4^+\)) toxicities (Britto and Kronzucker 2002; Kronzucker et al. 2006; ten Hoopen et al. 2010).

Since the pioneering work of Epstein et al. (1963), which described the acquisition of K\(^+\) by plants as the sum of activities of two transport systems with distinct substrate-binding affinities, major advances in the molecular and thermodynamic characterization of each system have been made (for review, see Maathuis and Sanders 1996; Véry and Sentenac 2003; Britto and Kronzucker 2008; Szczerba et al. 2009). Generally, Epstein’s mechanism 1, or the high-affinity transport system (HATS), has been described as a saturable system that catalyzes the thermodynamically active uptake (\textit{i.e.}, ATP-dependent transport against an electrochemical gradient) of K\(^+\) from external concentrations ([K\(^+\)]\(_{\text{ext}}\)) of less than 1 mM (Kochian and Lucas 1982; Maathuis and Sanders 1994). Mechanism 2, or the low-affinity transport system (LATS), catalyzes a flux proportional to [K\(^+\)]\(_{\text{ext}}\) and is proposed to predominate above 1 mM [K\(^+\)]\(_{\text{ext}}\) (Epstein et al. 1963; Kochian et al. 1985; Maathuis and Sanders 1996b). Although several
molecular candidates have been suggested to encode HATS and LATS proteins (Szczerba et al. 2009), it is believed that the majority of high-affinity transport is catalyzed by secondary active transporters of the HAK/KUP/KT family (e.g. AtHAK5 from Arabidopsis [Arabidopsis thaliana] and HvHAK1 from barley [Hordeum vulgare]), which operate via a proton (H⁺)/K⁺ symport mechanism (Gierth and Mäser 2007). Low-affinity transport, by contrast, occurs via Shaker-like K⁺ channels (e.g. AtAKT1 from Arabidopsis and HvAKT1 from barley), which facilitate passive diffusion down the electrochemical gradient for K⁺ (Very and Sentenac 2003; Cherel 2004).

Other key transporters implicated in K⁺ uptake include nonselective cation channels (NSCCs; Demidchik et al. 2002) and HKT/TRK-type transporters (Rubio et al. 1995). An unidentified system in Arabidopsis, independent of AtAKT1 and AtHAK5 and reportedly operating only at high (millimolar) [K⁺]_{ext}, has been the subject of considerable recent interest (Pyo et al. 2010; Rubio et al. 2010; Caballero et al. 2012; see also Hirsch et al. 1998); however, little is known about its molecular and physiological characterization.

The nutritional and molecular regulation of HAK/KUP/KT transporters and Shaker-like K⁺ channels has been extensively investigated in the model system Arabidopsis and has led to some blurring of distinctions between the traditional concepts of HATS and LATS (Spalding et al. 1999; Xu et al. 2006; Lee et al. 2007; Qi et al. 2008; Geiger et al. 2009; Honsbein et al. 2009; Pyo et al. 2010; Rubio et al. 2010; for review, see Alemán et al. 2011). For instance, although HAK/KUP/KT transporters appear to dominate the HATS (Gierth and Mäser 2007), some members have been demonstrated to operate at [K⁺]_{ext} as high as 20 mM, indicating a dual affinity (Fu and Luan 1998; Kim et al. 1998). By contrast, although generally ascribed to the LATS (Maathuis and Sanders, 1996), AtAKT1 can operate at [K⁺]_{ext} as low as 10 µM, given favorable thermodynamic conditions, and particularly if the HATS is suppressed by NH₄⁺ (Hirsch et al. 1998; Spalding et al. 1999), which specifically inhibits HAK/KUP/KT transporters (Qi et al. 2008). Further blurring the distinction between HATS and LATS is the response of K⁺ uptake mechanisms to K⁺ limitation (Hampton et al. 2004). For instance, HAK/KUP/KT expression (Ahn et al. 2004; Gierth et al. 2005) and high-affinity K⁺ influx (Glass 1976; Kochian and Lucas 1982; Siddiqi and Glass 1986) have both been shown to be upregulated by K⁺ starvation. On the other hand, while AtAKT1 expression appears to be independent of K⁺ availability (Lagarde et al. 1996; Gierth et al. 2005), the posttranslational regulation of AtAKT1 (including phosphorylation/dephosphorylation networks and channel heteromerization) has
shown that channel-mediated K⁺ uptake can increase in response to low-K⁺ conditions (Li et al. 2006; Xu et al. 2006; Lee et al. 2007; Geiger et al. 2009; Grefen et al. 2010; Jeanguenin et al. 2011).

While much recent work on K⁺ uptake has occurred in Arabidopsis, leading to the development of a sophisticated model (Alemán et al. 2011), little attention has been given to the question of how general this model might be, most importantly with respect to crop species. This is naturally the result of the vast catalog of well-characterized Arabidopsis mutant lines (e.g. The Arabidopsis Information Resource [http://www.arabidopsis.org] and the Arabidopsis Biological Resource Center [http://abrc.osu.edu]), which have no equivalent in crop species such as rice (Oryza sativa; Goff et al. 2002; Yu et al. 2002; see also Amrutha et al. 2007), barley (Mayer et al. 2012), and wheat (Triticum aestivum; Brenchley et al. 2012). Examinations into the molecular identity and contribution of K⁺ uptake systems in roots of barley have been limited to high-affinity concentrations (0.1–1 mM), with particular focus on the AtHAK5 homolog, HvHAK1, and its regulation (Santa-María et al. 2000; Vallejo et al. 2005; Fulgenzi et al. 2008). This work indicates that, like Arabidopsis (Hirsch et al. 1998; Spalding et al. 1999), high-affinity K⁺ uptake in roots of barley is dictated by NH₄⁺-sensitive and -insensitive systems, linked to HvHAK1 and, most likely, HvAKT1, respectively (Santa-María et al. 2000). However, it has not been explored whether, under high-NH₄⁺ conditions, K⁺ channels can operate in roots of barley at very low [K⁺]ₑₓ (e.g. 10 µM), as they do in Arabidopsis (Hirsch et al., 1998); this information can help address some longstanding speculation on the role of K⁺ channels under nutrient deficiency (Kochian and Lucas 1993). Moreover, the relative apportionment of K⁺ channels and secondary-active transporters with respect to high- and low-affinity uptake in roots of barley has not been explored to the same extent as in Arabidopsis (Rubio et al. 2008, 2010). Lastly, although the inhibition of high-affinity K⁺ influx by external NH₄⁺ supply is well documented (Vale et al. 1987; Spalding et al. 1999; Santa-María et al. 2000; Qi et al. 2008), as are its effects on membrane polarization (Ullrich et al. 1984; Wang et al. 1994; Britto et al. 2001b), little is known about the recovery of K⁺ influx and the thermodynamic response following NH₄⁺ removal under high- and low-affinity systems. As one of the chief aspects of NH₄⁺ toxicity in higher plants, understanding the inhibitory role of this nitrogen source in K⁺ transport is of particular importance (Britto and Kronzucker 2002).
Here, we address these gaps in understanding by providing, to our knowledge, the first in-depth physiological examination of the contribution of K⁺ channels and high-affinity transporters to K⁺ acquisition in barley. In particular, we posed the following questions. What is the relative apportionment of K⁺ channels and high-affinity transporters to high- and low-affinity K⁺ uptake in the presence of NH₄⁺ in barley, and how does it differ from the Arabidopsis model? What are the maximal rates of high- and low-affinity K⁺ fluxes *in planta*? How does K⁺ uptake respond to NH₄⁺ withdrawal, and what mechanisms underlie this response? With the use of ⁴²K⁺ radiolabeling, coupled with mutant and electrophysiological analyses, we show that K⁺ acquisition at low (22.5 µM) [K⁺]₇, in the presence of high (millimolar) NH₄⁺, is fundamentally different in the two model systems, chiefly in that K⁺ channels operate at such low [K⁺]₇ in Arabidopsis but not in barley. However, we show that with sudden withdrawal of external NH₄⁺, dramatic shifts in thermodynamic gradients and K⁺ fluxes can occur, revealing novel aspects of transport capacity and plasticity. We also provide, to our knowledge, the first *in planta* ⁴²K⁺ examination of the *athak5 atakt1* double mutant, revealing novel aspects of an uptake system as yet unidentified by genetic means.

### 4.1.3 Results

*The Relative Apportionment of K⁺ Channels and High-Affinity Transporters Differs Between Barley and Arabidopsis under Steady-State Conditions*

Figure 12 shows the results of an extensive pharmacological profiling of steady-state K⁺ influx, targeting either Shaker-like K⁺ channels and HAK/KUP/KT transporters (Fig. 12A, C, and E) or NSCCs (Fig. 12B, D, and F), in barley grown and tested under high (10 mM) NH₄⁺ and three levels of [K⁺]₇: 0.0225 mM (low), 0.1125 mM (intermediate), and 5 mM (high). K⁺ influx was insensitive to the standard K⁺ channel inhibitors, tetraethyl ammonium (TEA⁺) and Ba²⁺ (White and Lemtiri-Chlieh 1995; Bertl et al. 1997; Hille 2001), under low-K⁺ conditions (Fig. 12A) but showed significant (P <0.05) inhibition at intermediate K⁺ (Fig. 12C). Under high-K⁺ conditions, influx was suppressed by TEA⁺ and, surprisingly, stimulated by BaCl₂ (Fig. 12E), as also observed with 5 mM CaCl₂ and Ca(NO₃)₂ (Fig. 12F). Cs⁺, a potent inhibitor of both K⁺ channels and high-affinity transporters (Krol and Trebacz 2000; White and Broadley 2000), significantly suppressed K⁺ influx at low, intermediate, and high K⁺, by 41%, 81%, and 57%, respectively.
(Fig. 12A, C, and E). Metabolic inhibitors vanadate (VO$_4^{3-}$), 2,4-dinitrophenol (DPN), diethylstilbestrol (DES), cyanide (CN$^-$) + salicylhydroxamic acid (SHAM), carbonyl cyanide $m$-chlorophenyl hydrazone (CCCP), and pH 9.2 (adjusted with NaOH) were all very effective in suppressing K$^+$ influx under all [K$^+$]$_{ext}$ conditions tested, except for DNP at low K$^+$ (Fig. 12A, C, and E). The NSCC inhibitors Ca$^{2+}$, Gd$^{3+}$, La$^{3+}$, Glu, and diethylpyrocarbonate (DEPC; White and Lemtiri-Chlieh 1995; Essah et al. 2003) had no effect on K$^+$ influx at any [K$^+$]$_{ext}$ tested (Fig. 12B, D, and F). Note that counter-ion controls for VO$_4^{3-}$, CN$^-$, Glu, and pH 9.2 treatments were conducted with 10 mM NaCl and showed no response at any [K$^+$]$_{ext}$ (data not shown).

Table 7 displays the results of a thermodynamic (Nernstian) analysis for barley based on compartmental analysis by $^{42}$K$^+$ efflux and electrophysiology. Since physiological efflux was confirmed for low- and intermediate-K$^+$ conditions (Coskun et al. 2010; see below), the methods of compartmental analysis (Lee and Clarkson 1986; Siddiqi et al. 1991; Kronzucker et al. 1995) were used to estimate cytosolic potassium concentration ([K$^+$]$_{cyt}$), along with unidirectional fluxes and cytosolic halftimes of exchange. Based on estimates of [K$^+$]$_{cyt}$, equilibrium potentials for K$^+$ ($E_{K^+}$) were calculated (see Chapter 7, “Materials and Methods”) and were found to be more negative than measured membrane potentials ($\Delta \Psi_m$) for epidermal and cortical root cells at both K$^+$ conditions (Table 7); thus, thermodynamically active K$^+$ uptake was predicted. Since physiological efflux was not found under high-K$^+$ conditions (see below), neither compartmental nor subsequent thermodynamic analyses could be conducted under those conditions. We should note that $E_{K^+}$ is only an approximation, as it should use K$^+$ activities rather than K$^+$ concentrations. However, approximations of cytosolic K$^+$ activity coefficients ($\gamma_{cyt}$) vary widely in the literature (e.g. 0.72 < $\gamma_{cyt}$ < 1.29; Kielland 1937; Robinson and Stokes 1965; Ling 1969; Palmer et al. 1978), reflecting a still surprisingly poor understanding of ion sequestration and interaction in the plant cell cytosol (Cheeseman 2013). When K$^+$ activities were estimated according to standard procedures (using $\gamma_{cyt}$ = 0.75; Walker et al. 1996a; Cuin et al. 2003), however, $E_{K^+}$ still remained negative of $\Delta \Psi_m$ (data not shown); thus, the principal thermodynamic conclusions of our analysis still hold.

A selective pharmacological profiling of K$^+$ influx in Arabidopsis (Columbia [Col-0] wild type, Wassilewskija [WS] wild type, $trak1$, $athak5$, and $athak5$ $trak1$) was conducted at low K$^+$ and high NH$_4^+$ (Fig. 13). Because Arabidopsis exhibited NH$_4^+$ toxicity at much lower concentrations than barley (data not shown), NH$_4^+$ was provided only at 2 mM (compared with 10 mM in

66
barley). Unlike barley, Arabidopsis wild-type lines generally displayed TEA$^+$ and Ba$^{2+}$ sensitivity under low-K$, high-NH$_4$ conditions (although it was not statistically significant in the case of TEA$^+$ for WS; Fig. 13B). Consistent with their pharmacological targeting (see above), TEA$^+$ and Ba$^{2+}$ sensitivity was not found in the AtAKT1 knockout lines atakt1 and athak5 atakt1 (Fig. 13C and E). Further confirmation of the involvement of AtAKT1 was provided by the dramatic decrease in steady-state influx in atakt1 and athak5 atakt1 lines compared with their respective wild types (59% and 78%, respectively), while influx for athak5 was essentially equal to the Col-0 wild type and displayed both TEA$^+$ and Ba$^{2+}$ sensitivity (albeit not statistically significant in the case of TEA$^+$; Fig. 13D). Cs$^+$ significantly suppressed steady-state influx in all lines, including athak5 atakt1 (Fig. 13A–D), while pH 9.2 was about as effective as Cs$^+$ in all cases except in athak5 atakt1 (Fig. 13A–D). Ca$^{2+}$ was ineffective at suppressing steady-state influx in all lines (Fig. 13A–D).
**Figure 12.** The effects of various pharmacological and nutritional treatments, targeting either Shaker-like K⁺ channels and HAK/KUP/KT transporters (A, C, and E) or NSCCs (B, D, and F), on steady-state K⁺ influx in intact roots of barley seedlings grown on a full-nutrient medium at low (A and B), intermediate (C and D), and high (E and F) [K⁺]_{ext} and 10 mM [NH₄⁺]_{ext}. Fluxes are indicated on a root fresh weight basis. Asterisks denote different levels of significance between control and treatment pairs (*0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001; one-way ANOVA with Dunnett’s multiple comparison post-hoc test). Each treatment represents the mean of four to 69 replicates. Error bars indicate SE.
Table 7. Steady-state K⁺ fluxes, compartmentation, and electrophysiology of intact barley seedling roots. One-week-old barley seedlings were grown on a full-nutrient medium supplemented with 10 mM NH₄⁺ and either 0.0225 or 0.1125 mM K⁺. ΔΨₘ measurements were taken from root epidermal and cortical cells 2 to 3 cm from the root tip. E_K⁺ and predicted [K⁺]ₗᵢₜ were determined with the Nernst equation. Error values indicate SE of six to eight replicates.

<table>
<thead>
<tr>
<th>[K⁺]ₑₓᵗ (mM)</th>
<th>Influx (µmol g⁻¹ h⁻¹)</th>
<th>Efflux (µmol g⁻¹ h⁻¹)</th>
<th>Net flux (µmol g⁻¹ h⁻¹)</th>
<th>E:I Ratio</th>
<th>t₁/₂ (min)</th>
<th>ΔΨₘ (mV)</th>
<th>E_K⁺ (mV)</th>
<th>[K⁺]ₗᵢₜ (mM)</th>
<th>Predicted</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0225</td>
<td>0.52 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.52 ± 0.03</td>
<td>32.90 ± 2.92</td>
<td>-143.6 ± 2.7</td>
<td>-153.8</td>
<td>5.57</td>
<td>8.24 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>0.1125</td>
<td>1.89 ± 0.13</td>
<td>0.57 ± 0.05</td>
<td>1.32 ± 0.10</td>
<td>0.30 ± 0.01</td>
<td>32.50 ± 4.69</td>
<td>-136.2 ± 3.0</td>
<td>-144.1</td>
<td>20.95</td>
<td>28.39 ± 3.40</td>
<td></td>
</tr>
</tbody>
</table>
Figure 13. The effects of various pharmacological and nutritional treatments on steady-state K⁺ influx in intact roots of Arabidopsis Col-0 wild type (A), WS wild type (B), atakt1 (C), athak5 (D), and athak5 atakt1 (E) grown on a full-nutrient medium at low [K⁺]_{ext} and 2 mM [NH₄⁺]_{ext}. Fluxes are indicated on a root fresh weight basis. Asterisks denote different levels of significance between control and treatment pairs (*0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001; one-way ANOVA with Dunnett’s multiple comparison post-hoc test). Each treatment represents the mean of three to 14 replicates. Error bars indicate SE.
Mechanism of $K^+$ Efflux in Barley Differs Based on $[K^+]_{ext}$

Figure 14 illustrates the pharmacological profiling of steady-state $K^+$ efflux in barley under high NH$_4^+$ and varying $[K^+]_{ext}$. Interestingly, at low (22.5 µM) $[K^+]_{ext}$, application of 10 mM Cs$^+$ resulted in an immediate and significant stimulation in $K^+$ efflux (Fig. 14A), unlike TEA$^+$, Ba$^{2+}$, and Ca$^{2+}$, which had no effect. By contrast, at intermediate (112.5 µM) $[K^+]_{ext}$, Cs$^+$ was effective in inhibiting efflux, as was TEA$^+$ (Fig. 14B). At high (5 mM) $[K^+]_{ext}$, Cs$^+$ showed no effect (Fig. 14C), consistent with the cessation of physiological efflux, which was confirmed by comparing steady-state efflux to pH 9.2 treatments, where roots had been exposed to alkalinity during tracer uptake and elution periods (see Chapter 7, “Materials and Methods”). Since pH 9.2 was effective in suppressing influx at all $[K^+]_{ext}$ tested (Fig. 12A, C, and E), its application during tracer uptake would have inhibited intracellular accumulation. Thus, the significant suppression of tracer release during the slowly exchanging phase in the low- and intermediate- $K^+$ conditions, and lack thereof at high $K^+$ (Fig. 14, insets), with pH 9.2 indicates the cytosolic origin of released tracer at low and intermediate $K^+$ and the lack thereof at high $K^+$. Sudden withdrawal of external NH$_4^+$ resulted in thermodynamic shifts (at low and intermediate $K^+$) and significant stimulations in $K^+$ influx (see below), but it was also found to inhibit $K^+$ efflux at both $[K^+]_{ext}$ and to cause no effect at high $K^+$ (Fig. 14, insets), confirming the proposed dual nature of efflux under high- and low-affinity conditions.
Figure 14. Responses of $^{42}\text{K}^+$ efflux from roots of intact barley seedlings to sudden application (see arrows) of various pharmacological and nutritional treatments. Plants were grown on a full-nutrient medium at low (A), intermediate (B), and high (C) $[\text{K}^+]_{\text{ext}}$ and 10 mM $[\text{NH}_4^+]_{\text{ext}}$. Insets show responses of $\text{K}^+$ efflux to sudden (see arrows) withdrawal of external $\text{NH}_4^+$ and/or alkalinity during radiotracer uptake and elution periods. Numbers in parentheses indicate percentage of treated points differing significantly from control (Student’s $t$ test; $P < 0.05$). In the insets, axis labels are as in the main figures. Each plot represents the mean of three to seven replicates. Error bars indicate SE.
NH₄⁺ Withdrawal Results In Thermodynamic Shifts and Significant K⁺ Influx

K⁺ influx was stimulated by sudden withdrawal (5-min pretreatment) of NH₄⁺, by 176%, 355%, and 131% (corresponding to 2.4 ± 0.1, 12.0 ± 0.4, and 24.3 ± 0.7 µmol g⁻¹ (root fresh weight) h⁻¹, respectively), at low-, intermediate-, and high-K⁺ conditions, respectively (Fig. 15). NH₄⁺ withdrawal also led to immediate and significant hyperpolarization of root epidermal and cortical ΔΨₘ (59.1 ± 13.2, 53.1 ± 8.3, and 31.4 ± 4.9 mV at low, intermediate, and high K⁺, respectively), corresponding to ΔΨₘ much more negative than E_K⁺ and, thus, thermodynamic shifts (active to passive influx) at low and intermediate K⁺ (Table 8; compare with Table 7).

This ammonium withdrawal effect (AWE) in barley was also found in Arabidopsis wild-type lines, resulting in 142% and 175% increases in K⁺ influx for Col-0 and WS, respectively (Fig. 16A and B). Interestingly, AWE was also consistently observed in the knockout lines atakt1 (513%), athak5 (167%), and athak5 atakt1 (32% [albeit not statistically significant]; Fig. 16C–E). As in barley, NH₄⁺ withdrawal also consistently resulted in hyperpolarization of the plasma membrane of root epidermal and cortical cells in all lines except athak5 atakt1 (albeit not statistically significant in the cases of WS and athak5; Table 8).
**Figure 15.** The effects of various pharmacological and nutritional treatments on K⁺ influx stimulated due to NH₄⁺ withdrawal in intact roots of barley seedlings grown on a full-nutrient medium at low (A), intermediate (B), and high (C) [K⁺]ₐₜ and 10 mM [NH₄⁺]ₐₜ. Fluxes are indicated on a root fresh weight basis. Asterisks denote different levels of significance between –NH₄⁺ and treatment pairs (*0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001; one-way ANOVA with Dunnett’s multiple comparison post-hoc test). Asterisks in parentheses denote levels of significance between control and –NH₄⁺ pairs (Student’s t test). Each treatment represents the mean of four to 69 replicates. Error bars indicate SE.
Table 8. Electrophysiological responses of barley and Arabidopsis root cells to sudden NH$_4^+$ withdrawal with or without Ca(NO$_3$)$_2$. One-week-old barley seedlings and 5-week-old Arabidopsis plants were grown on a full-nutrient medium supplemented with high NH$_4^+$ (2 mM in Arabidopsis, 10 mM in barley) and 0.0225, 0.1125, or 5 mM K$^+$. ΔΨ$_m$ measurements were taken from root epidermal and cortical cells 2 to 3 cm from the root tip, and the AWE + Ca(NO$_3$)$_2$ (5 mM in barley) was measured. Letters denote significantly different means (P < 0.05; one-way ANOVA with Bonferroni post-hoc test), and asterisks denote different levels of significance between control and treatment pairs (ns, not significant; **0.001 < P < 0.01; Student’s t test). Error values indicate SE of four replicates. n.d., not determined.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>$[K^+]_{ext}$ (mM)</th>
<th>ΔΨ$_m$ (mV)</th>
<th>Control</th>
<th>AWE</th>
<th>AWE + Ca(NO$_3$)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>barley</td>
<td>Metcalfe (wild type)</td>
<td>0.0225</td>
<td>-143.6 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-202.6 ± 21.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-251.4 ± 13.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1125</td>
<td>-136.2 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-189.3 ± 8.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-208.5 ± 10.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>-133.2 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-164.6 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-149.0 ± 8.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Col-0</td>
<td>0.0225</td>
<td>-165.0 ± 13.7</td>
<td>-231.0 ± 8.1**</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WS</td>
<td></td>
<td>-137.6 ± 7.2</td>
<td>-172.9 ± 21.5&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>atakt1</td>
<td></td>
<td>-139.2 ± 3.5</td>
<td>-192.4 ± 8.3**</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>athak5</td>
<td></td>
<td>-106.1 ± 8.6</td>
<td>-156.6 ± 22.2&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>athak5 atakt1</td>
<td></td>
<td>-258.0 ± 22.9</td>
<td>-265.3 ± 20.8&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>
Ca\textsuperscript{2+} Sensitivity of the AWE Depends on [K\textsuperscript{+}]_{ext} and Genotype

The results of a selective pharmacological profiling of AWE in barley can be seen in Figure 15. Under the three levels of [K\textsuperscript{+}]_{ext}, AWE displayed similar inhibitory responses to TEA\textsuperscript{+}, Ba\textsuperscript{2+}, and Cs\textsuperscript{+} (i.e. TEA\textsuperscript{+} < Ba\textsuperscript{2+} < Cs\textsuperscript{+}). Interestingly, AWE also displayed significant (P < 0.01) inhibition by 5 mM Ca\textsuperscript{2+} (regardless of the counter ion) at low and intermediate K\textsuperscript{+} (Fig. 15A and B) but not at high K\textsuperscript{+} (Fig. 15C). To the contrary, AWE at high K\textsuperscript{+} was stimulated by Cl\textsuperscript{−} and NO\textsubscript{3}\textsuperscript{−} (supplied as Ca\textsuperscript{2+} salts), with the greatest stimulation observed with NO\textsubscript{3}\textsuperscript{−} [36 µmol g\textsuperscript{−1} (root fresh weight) h\textsuperscript{−1}]. AWE showed no response to the NSCC inhibitors DEPC, Gd\textsuperscript{3+}, and La\textsuperscript{3+} at any [K\textsuperscript{+}]_{ext} (Fig. 15).

The differential response of AWE to Ca(NO\textsubscript{3})\textsubscript{2} under varying [K\textsuperscript{+}]_{ext} (i.e. inhibition at low and intermediate K\textsuperscript{+}, stimulation at high K\textsuperscript{+}) was also explored electrophysiologically. As shown in Table 8, switching from (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to equimolar Ca(NO\textsubscript{3})\textsubscript{2} resulted in further hyperpolarization of the plasma membrane of root epidermal and cortical cells at low and intermediate K\textsuperscript{+} compared with NH\textsubscript{4}\textsuperscript{+} withdrawal alone. By contrast, at high K\textsuperscript{+}, this resulted in less hyperpolarization compared with NH\textsubscript{4}\textsuperscript{+} withdrawal alone. Similar to barley (at low K\textsuperscript{+}), AWE in Arabidopsis wild-type lines showed comparable responses to TEA\textsuperscript{+}, Ba\textsuperscript{2+}, Cs\textsuperscript{+}, and Ca\textsuperscript{2+} (Fig. 16A and B). This was also observed in athak5 (Fig. 16D). As expected, AWE in atakt1 and athak5 atakt1 no longer displayed sensitivity to the channel inhibitors TEA\textsuperscript{+} and Ba\textsuperscript{2+} but remained significantly Cs\textsuperscript{+} sensitive (Fig. 16C and E). Surprisingly, however, the Ca\textsuperscript{2+} sensitivity of AWE was lost in atakt1 and athak5 atakt1 (Fig. 16C and E).
**Figure 16.** The effects of various pharmacological and nutritional treatments on K⁺ influx stimulated due to NH₄⁺ withdrawal in intact roots of Arabidopsis Col-0 wild type (A), WS wild type (B), atakt1 (C), athak5 (D), and athak5 atakt1 (E) grown on a full-nutrient medium at low [K⁺]_{ext} and 2 mM [NH₄⁺]_{ext}. Fluxes are indicated on a root fresh weight basis. Asterisks denote different levels of significance between –NH₄⁺ and treatment pairs (*0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001; one-way ANOVA with Dunnett’s multiple comparison post-hoc test). Asterisks in parentheses denote levels of significance between control and –NH₄⁺ pairs (Student’s t test). Each treatment represents the mean of four to 14 replicates. Error bars indicate SE.
AWE over 24 h Reveals Peaks in $K^+$ Influx and Leads to Tissue $K^+$ Accumulation

Figure 17 illustrates the sustainability of AWE (i.e. elevated $K^+$ influx after ammonium withdrawal) and its magnitude over 24 h at all three $K^+$ levels in barley. At low $K^+$, AWE was not only sustained over 24 h but continued to rise, reaching 4.2 µmol g$^{-1}$ (root fresh weight) h$^{-1}$ (a 4.6-fold increase from control). At intermediate $K^+$, AWE plateaued between 8 and 12 h, reaching 10 µmol g$^{-1}$ (root fresh weight) h$^{-1}$ (a 6.8-fold increase from control), before dropping to approximately 5 µmol g$^{-1}$ (root fresh weight) h$^{-1}$ by 24 h. At high $K^+$, AWE peaked at 25 µmol g$^{-1}$ (root fresh weight) h$^{-1}$ by 1 h before leveling off at approximately 10 µmol g$^{-1}$ (root fresh weight) h$^{-1}$ by the end of the 24-h period. Despite variations in the sustainability of AWE (Fig. 17), NH$_4^+$ withdrawal consistently resulted in increased tissue (shoot, root, and total) $K^+$ accumulation over 24 h at all $K^+$ levels tested. By the end of 24 h, NH$_4^+$ withdrawal resulted in 136%, 150%, and 27% increases in total tissue $K^+$ levels under low-, intermediate-, and high-$K^+$ conditions, respectively (Fig. 18). Interestingly, NH$_4^+$ withdrawal over 24 h also resulted in increased tissue accumulation of Na$^+$ (Fig. S1).
Figure 17. Responses of K⁺ influx to the duration of NH₄⁺ withdrawal in roots of intact barley seedlings grown on a full-nutrient medium, various [K⁺]_{ext} levels, and 10 mM [NH₄⁺]_{ext}. Fluxes are indicated on a root fresh weight basis. Each data point represents the mean of four replicates. Error bars indicate SE.
**Figure 18.** Responses of tissue-$K^+$ content to the duration of $\text{NH}_4^+$ withdrawal in barley seedlings grown on a full-nutrient medium at low (A), intermediate (B), and high (C) $[K^+]_{\text{ext}}$ and 10 mM $[\text{NH}_4^+]_{\text{ext}}$. Content measurements are indicated on a root fresh weight basis. Each data point represents the mean of four replicates. Error bars indicate SE.
4.1.4 Discussion

Although the genetic identities of K\(^+\) channels and high-affinity transporters, and their contributions to high- and low-affinity K\(^+\) uptake, have been examined extensively in Arabidopsis, few studies have placed these fundamental discoveries into the context of agriculturally important species such as barley. In this work, both barley and Arabidopsis were grown at high external ammonium concentration ([NH\(_4^+\)]\(_{\text{ext}}\) of 10 and 2 mM, respectively; the difference a reflection of each species’ unique sensitivity to NH\(_4^+\); Britto and Kronzucker 2002; ten Hoopen et al. 2010) to inhibit high-affinity transporters and thereby isolate K\(^+\)-channel functioning (Hirsch et al. 1998; Spalding et al. 1999; Santa-María et al. 2000). We should also note that each species was grown for different lengths of time in different media (see Chapter 7, “Materials and Methods”), reflecting their different developmental programs and nutritional preferences. Although differences in growth regime and developmental stage are important to consider, both species displayed severely reduced high-affinity K\(^+\) uptake due to NH\(_4^+\) (see below), and this set the stage for critical evaluations and comparisons of K\(^+\)-transporter functioning. At the lowest [K\(^+\)]\(_{\text{ext}}\) tested (0.0225 mM), fundamental differences in the apparent mechanisms of steady-state K\(^+\) uptake were observed between the two species (Fig. 19). In both the wild type and athak5 mutants of Arabidopsis, K\(^+\) influx was blocked by the classic channel inhibitors TEA\(^+\) and Ba\(^{2+}\) (Fig. 13A, B, and D), indicating the involvement of the Shaker-like K\(^+\) channel AtAKT1 at very low [K\(^+\)]\(_{\text{ext}}\) in this species (and confirming the results of Hirsch et al. [1998] and Spalding et al. [1999]). Moreover, the AtAKT1 knockout lines atakt1 and athak5 atakt1 showed significantly lower (59% and 78%) influx than their respective wild types. By contrast, in barley, neither TEA\(^+\) nor Ba\(^{2+}\) affected K\(^+\) influx at this [K\(^+\)]\(_{\text{ext}}\) (Fig. 12A), indicating that it does not appear to involve (TEA\(^+\)- or Ba\(^{2+}\)-sensitive) channels; this is consistent with our thermodynamic (Nernstian) analysis based on compartmental and electrophysiological data, which predicted influx to be an active process (Table 7). Consistent with this analysis, influx in barley was significantly suppressed by the K\(^+\)-transporter inhibitor Cs\(^+\), the metabolic inhibitors VO\(_4^{3-}\), DES, CN\(^-\) + SHAM, and CCCP, and pH 9.2 (which collapses the electrochemical potential difference for protons). Thus, it appears that steady-state potassium influx at low K\(^+\) and high NH\(_4^+\), while reduced, is mediated by high-affinity transporters in this species, most likely including HvHAK1 (Santa-María et al. 1997). We should note, however, that pH 9.2 also inhibited steady-state influx in athak5 (Fig. 13D), indicating the possibility of alkalinity-induced AtAKT1 inhibition (Fuchs et al. 2005).
Interestingly, the Cs⁺ sensitivity of K⁺ influx in athak5 atakt1 double mutants at low K⁺ suggests that a genetically unidentified uptake system is operative, albeit at a relatively minor capacity. This is in contrast to other reports suggesting that “unknown systems” only operate at high [K⁺]_{ext} (more than 0.5 mM; Pyo et al. 2010; Rubio et al. 2010). Thus, at least two distinct unknown systems appear to operate in Arabidopsis; moreover, the pharmacological profile of athak5 atakt1 in our study did not match that of others, particularly with respect to Ca²⁺ and Ba²⁺ sensitivity (Caballero et al. 2012). This raises the question of whether similar mechanisms are at play in barley at low-K⁺, high-NH₄⁺ conditions and provides avenues for future research.

At [K⁺]_{ext} above 0.1 mM, by contrast, K⁺ channels do appear to participate in K⁺ uptake in barley, since, at the intermediate [K⁺]_{ext} tested (0.1125 mM), we observed significant inhibition of K⁺ influx by TEA⁺ and Ba²⁺ (Fig. 12C). Although thermodynamic analyses suggested K⁺ uptake at intermediate K⁺ to be an active process (Table 7), this apparent contradiction most likely reflects some methodological discrepancies. Pharmacological testing of K⁺ influx and compartmental analysis of ^{42}K⁺ efflux take the entire root into account and thus reflect an average of different cell types along the root axis. On the other hand, electrophysiological measurements of ΔΨₘ are, by nature, single-cell measurements and thus do not necessarily represent the whole root. In fact, membrane polarization is known to follow the longitudinal axis of the root, with the most polarized cells located near the root tip (Hirsch et al. 1998). As our ΔΨₘ measurements were made 2 to 3 cm from the root tip (see Chapter 7, “Materials and Methods”), it is likely that we measured from cells not polarized enough to conduct passive K⁺ uptake. Thus, at intermediate [K⁺]_{ext}, what we observe may be a mixed population of cells: some conducting channel-mediated K⁺ uptake, others engaging high-affinity transporters. At 5 mM [K⁺]_{ext}, where channel-mediated K⁺ uptake is proposed to dominate and high-affinity transporters are assumed to be largely irrelevant (Maathuis and Sanders 1996b; Rubio et al. 2010), we did indeed find TEA⁺ sensitivity; however, oddly, we also observed stimulations in steady-state K⁺ influx with BaCl₂ application (Fig. 12E). Similarly, applications of CaCl₂ and Ca(NO₃)₂ also stimulated K⁺ influx (Fig. 12F), while they produced no effect at low and intermediate [K⁺]_{ext} (Fig. 12B and D, respectively). This may be the result of anion effects specific to the LATS, as demonstrated in earlier reports (Epstein et al. 1963; Kochian et al. 1985). Kochian et al. (1985) showed that Cl⁻ stimulated low-affinity K⁺ uptake (possibly via coupled transport), unlike SO₄^{2⁻}, H₂PO₄⁻, and (in contrast to this study) NO₃⁻. The apparent contradiction with respect to
\[\text{NO}_3^-\] could be attributed to a variety of differences in experimental procedures, including species, growth medium, and influx protocol. Surprisingly, further elucidation of the mechanism underlying this effect has not occurred since then; however, this falls out of the scope of our study. What is important to note is that three distinct uptake scenarios appear to occur in barley roots grown under high \(\text{NH}_4^+\) and varying \([\text{K}^+]_{\text{ext}}\): at low \(\text{K}^+\), an active process dominates, attributable to residual \(\text{HvHAK1}\) activity and/or unknown systems; at intermediate \(\text{K}^+\), a mixed population of channel (\(\text{HvAKT1}\))- and high-affinity transporter (\(\text{HvHAK1}\))-mediated \(\text{K}^+\) uptake occurs; and at high \(\text{K}^+\), channel (\(\text{HvAKT1}\))-mediated \(\text{K}^+\) uptake appears to dominate, but with unique \(\text{Cl}^-\) and \(\text{NO}_3^-\)-induced stimulations (Fig. 19).

Consistent across all \([\text{K}^+]_{\text{ext}}\) tested in barley is the significant reduction of \(\text{K}^+\) influx by metabolic inhibitors (Fig. 12A, C, and E). Typically, the HATS is reported to be more sensitive to metabolic inhibitors compared with the LATS (Malhotra and Glass 1995); however, the vast majority of metabolic inhibitors was most effective in suppressing influx at the intermediate \(\text{K}^+\) level and not at the lowest level. Interestingly, influx at intermediate \(\text{K}^+\) was most strongly inhibited by \(\text{Cs}^+\) and was the most stimulated in response to \(\text{NH}_4^+\) withdrawal. Perhaps this is indicative of the mixed population of channels and high-affinity transporters operating under these conditions and reflects a highly dynamic transport capacity. Also evident is the lack of NSCC involvement at all \([\text{K}^+]_{\text{ext}}\) tested, as \(\text{Ca}^{2+}, \text{Gd}^{3+}, \text{La}^{3+}, \text{Glu}, \text{and DEPC}\) had no effect on \(\text{K}^+\) influx (Fig. 12B, D, and F; Essah et al. 2003). It is rather surprising that, if NSCCs catalyze high rates of cation fluxes, as has been proposed (White and Davenport 2002; Essah et al. 2003; Kronzucker and Britto 2011, and references therein), we were unable to observe their activity physiologically. However, the presence of significant concentrations of \(\text{Ca}^{2+}(1–5\text{ mM}),\) reflective of common soil conditions (Garcia-deblas et al. 2003; Zhang et al. 2010), may have reduced putative \(\text{K}^+\) currents through NSCCs considerably (White and Davenport 2002). Similarly, \(\text{Na}^+-\text{K}^+\) cotransport does not appear to be operative, as no effect of 10 mM \(\text{NaCl}\) on \(\text{K}^+\) influx was observed (data not shown), ruling out the involvement of HKT/TRK-type transporters (Rubio et al. 1995). It is worth highlighting here that pharmacological profiling, like any experimental method, is not without its caveats. Although a traditionally useful tool for gaining insight into the structure and function of membrane transporters, particularly \textit{in planta}, the lack of specificity of several blockers/chemical treatments (White and Broadley 2000; Coskun et al. 2012), as well as the need to employ relatively high concentrations at times, can potentially have
secondary (“pleiotropic”) effects. This by no means invalidates the use of pharmacology but simply speaks to the importance of a multipronged approach in such studies.

As with influx, efflux analysis in barley demonstrates that distinct mechanisms are at play at each of the three [K+]ext tested (Fig. 19). At low K+, where influx was determined to be solely active, we see the perplexing result of efflux stimulation upon Cs+ application (Fig. 14A). Testing of the involvement of Cs+-induced depolarizations in this phenomenon (Nocito et al. 2002) yielded negative results (data not shown). We should note that instances of cellular K+/Cs+ exchange have been documented in the animal literature (Beauge et al. 1973; Guerin and Wallon 1979); however, to our knowledge, no precedence exists in the plant literature. Moreover, this does not explain its isolated incidence at low [K+]ext; thus, the mechanism remains unknown. In stark contrast with these observations, both Cs+ and TEA+ significantly inhibited K+ efflux at intermediate K+ levels (Fig. 14B). This effect has also been observed at a similar [K+]ext (0.1 mM) under a NO3− background (Coskun et al. 2010) and is consistent with other reports of the Cs+ and TEA+ sensitivities of channel-mediated ion fluxes (Krol and Trebacz 2000; White and Broadley 2000). At the highest [K+]ext tested (5 mM), a third scenario emerged, one in which physiological efflux was ruled out, as was previously shown under low-affinity conditions with NO3−-grown barley (Coskun et al. 2010). While an examination of the mechanisms of K+ efflux was beyond the scope of this study, physiological efflux data at low and intermediate [K+]ext lend themselves to compartmental analysis (Lee and Clarkson 1986; Siddiqi et al. 1991; Kronzucker et al. 1995). In these cases, we have strong evidence that efflux at low and intermediate K+ is occurring from the cytosol and across the plasma membrane, allowing us to confidently estimate [K+]cyt (Table 7). The variability of these results also confirms some of the earlier work on barley by Kronzucker et al. (2003), which investigated the heterostasis of [K+]cyt, in particular its suppression on a high-NH4+ medium. The lack of physiological efflux at high K+ prevents us from making such estimates. Future studies will examine the differences in K+ efflux between NO3−- and NH4+-grown plants and may help provide insight into the mechanisms of NH4+ toxicity.

Withdrawal of NH4+ resulted in immediate and dramatic stimulations of K+ influx, as high as 4.5-fold in barley and 6-fold in Arabidopsis. We should note that there have been a few reports of K+ uptake stimulation upon NH4+ withdrawal in both barley (Santa-María et al. 2000) and Arabidopsis (Rubio et al. 2010); however, little attention was directed to this phenomenon. In
addition, the magnitude of unidirectional fluxes measured was minuscule in one study (approximately 1 µmol g⁻¹ (root fresh weight) h⁻¹; Santa-María et al. 2000) and not measured in the other (Rubio et al. 2010). By contrast, some of the fluxes recorded in this study at high K⁺ in barley (25–36 µmol g⁻¹ (root fresh weight) h⁻¹) are among the highest bona fide transmembrane fluxes of K⁺ reported. Although some published rates of unidirectional Na⁺ and NH₄⁺ fluxes under toxicity conditions are larger (e.g. 60–600 µmol g⁻¹ (root fresh weight) h⁻¹; Lazof and Cheeseman 1986; Britto et al. 2001b; Essah et al. 2003; Horie et al. 2007; Szczerba et al. 2008b), these values have come into question, particularly with respect to the improbably high energy costs of such fluxes (Britto and Kronzucker 2009). In this study, these powerful stimulations of K⁺ influx might be explained by the significant hyperpolarization observed upon NH₄⁺ withdrawal (Table 8). At low- and intermediate-K⁺ levels, these electrical changes translate into thermodynamic shifts from active to passive K⁺ uptake in barley, the latter driven by a powerful downhill gradient; this was confirmed by use of the channel-blocking agents TEA⁺, Ba²⁺, and Cs⁺ (Figs. 15 and 16). Interestingly, AWE was observed in both atakt1 and athak5 knockout lines (Fig. 16C and D) and was substantial in both cases (513% and 167% of control values, respectively), suggesting a sizable participation by both transporters. This result demonstrates that AtHAK5 can operate under thermodynamically passive conditions, lending support to the idea that some HAK/KUP/KT transporters have a dual-affinity nature (Fu and Luan 1998; Kim et al. 1998). However, it is not clear whether it would function under these conditions as a H⁺/K⁺ symporter (Gierth and Mäser 2007) or engage a channel-like mechanism (Fu and Luan 1998). Further experimentation is required to address this possibility. The fact that AWE was minor in athak5 atakt1 double mutants suggests that AtAKT1 and AtHAK5 are the major contributors, and any contribution from unknown systems is small (Fig. 16E), as it is under steady-state conditions (Fig. 13E). Lastly, the inhibition of K⁺ efflux upon NH₄⁺ withdrawal at low and intermediate K⁺ (Fig. 14A and B, insets) is further evidence for a shift in thermodynamic gradients; under these conditions, it is likely that physiological K⁺ efflux has shut down entirely.

Pharmacological profiling of AWE across all [K⁺]ext clearly implicated channel (AKT1) involvement; however, the response of AWE to external Ca²⁺ revealed some unusual results. At low and intermediate K⁺, AWE was all but suppressed by Ca²⁺ in barley (Fig. 15A and B). This effect was not observed at high K⁺, however: CaSO₄ had no effect, while CaCl₂ and Ca(NO₃)₂ both stimulated AWE, by 26% and 47%, respectively (Fig. 15C). The Ca²⁺ sensitivity of AWE
was also observed in Arabidopsis wild-type lines and athak5 mutants at low K⁺ (Fig. 16A, B, and D). Surprisingly, it was not observed in AtAKT1 knockout lines (Fig. 16C and E), suggesting that the Ca²⁺ sensitivity of AWE is linked to the AtAKT1 channel. To our knowledge, evidence of AtAKT1 blockage by external Ca²⁺ is sparse; in one case, though, a weak inhibition of inward-rectifying K⁺ channels in the plasma membrane of rye roots by high concentrations of Ca²⁺ was observed (White and Lemtiri-Chlieh 1995), which provides evidence that K⁺ channels, such as AtAKT1, may be sensitive to extracellular Ca²⁺ under some conditions. An alternative hypothesis, that Ca²⁺ co-provision coincides with an increased Ca²⁺ influx, resulting in less hyperpolarization and thus a reduced K⁺ flux, was ruled out in two ways. First, the Ca²⁺ channel inhibitor verapamil (Lee and Tsien 1983; cf. White 1998) was supplied alongside Ca²⁺ during NH₄⁺ withdrawal, with no effects observed (data not shown). Second, when ΔΨₐₘ was measured during Ca(NO₃)₂ co-provision with NH₄⁺ withdrawal, not only was the hyperpolarization undiminished, but, in fact, even greater hyperpolarization was measured (Table 8). Surprisingly, however, at high K⁺, membrane hyperpolarization was reduced, even though Ca(NO₃)₂ provision upon NH₄⁺ withdrawal resulted in even greater K⁺ influx than NH₄⁺ withdrawal alone (Table 8). Thus, it appears that AWE and its stimulation or inhibition do not solely depend on membrane polarization but involve other processes, such as channel gating (as with Ca²⁺ effects) or coupling to anion transport (as in the case of Cl⁻ and NO₃⁻ effects). This is further confirmed by the fact that the greatest AWE occurred in barley at intermediate K⁺ (355% increase from control) while the concomitant hyperpolarizations were no greater than that seen at other K⁺ levels (Table 8). Also, the largest relative AWE in Arabidopsis was seen in atakt1 (513%), but it, too, showed hyperpolarization no greater than in any other line (Table 8).

A 24-h time course revealed the upper limits of AWE on K⁺ influx, both in terms of magnitude and sustainability. At high K⁺, influx peaked at approximately 25 µmol g⁻¹ (root fresh weight) h⁻¹ by 1 h, in contrast with low and intermediate K⁺, at which the fluxes continued to rise over 24 and 8 h, respectively (Fig. 17). Nevertheless, all time courses resulted in increased accumulation of tissue K⁺, demonstrating the nutritional significance of this effect (Fig. 18). Interestingly, at low and intermediate K⁺, NH₄⁺ withdrawal also resulted in significant tissue accumulation of Na⁺ (Fig. S1), particularly in the roots. This may reflect the ability of Na⁺ to replace K⁺ in some of its cellular roles (Subbarao et al. 2003), since these growth conditions (low and intermediate [K⁺]ₐₖₚ, high [NH₄⁺]ₐₖₚ) are toxic for barley and result in extremely low tissue K⁺ levels (Fig. 18).
Some reports suggest that Na\(^+\) and K\(^+\) could share similar uptake mechanisms, such as K\(^+\) channels (for review, see Kronzucker and Britto 2011); thus, situations where stimulated channel activity is induced could account for these findings. While out of the scope of this work, it would be interesting to measure Na\(^+\) fluxes in such a scenario, especially in the toxic range, to better understand the mechanisms of Na\(^+\) transport.

We have demonstrated here that the Arabidopsis model of K\(^+\) acquisition is not universally applicable. Although K\(^+\) channels appear to be the sole means of K\(^+\) acquisition under low-K\(^+\), high-NH\(_4\)^+ conditions in Arabidopsis, this is not the case for barley. Our study provides new physiological evidence of three distinct modes of K\(^+\) influx and efflux in NH\(_4\)^+-grown barley, operating at 0.0225, 0.1125, and 5 mM [K\(^+\)]\(_{\text{ext}}\). Figure 19 illustrates these key findings.

Moreover, we demonstrate that NH\(_4\)^+ withdrawal can reveal a very high capacity and plasticity among K\(^+\) transporters. This work provides a framework of characteristics, including nutritional and pharmacological profiles, by which discoveries in molecular genetics, particularly in the emerging field of cereal genomics, can be gauged.
Figure 19. Schematic overview of K⁺ transport in plant roots under steady-state conditions (*i.e.* in the presence of high [millimolar] [NH₄⁺]ₜₐₐ₉). Under low (0.0225 mM) [K⁺]ₜₐ₉, K⁺ uptake is predominantly mediated by K⁺ channels (AtAKT1) in Arabidopsis (Col-0 wild type; A), whereas in barley (B), K⁺ channels do not operate and uptake is likely mediated by high-affinity transporters (HvHAK1), albeit at a residual capacity due to NH₄⁺-induced inhibition. Above intermediate K⁺ levels ([K⁺]ₜₐ₉ = 0.1125 mM), K⁺ channels do operate in barley, with further Cl⁻ and NO₃⁻-induced stimulations of K⁺ uptake observed at high K⁺ ([K⁺]ₜₐ₉ = 5 mM). In barley, K⁺ efflux is likely channel mediated at low and intermediate K⁺, although with varying sensitivities to Cs⁺, whereas K⁺ efflux is likely inoperative at high K⁺. K⁺ efflux in Arabidopsis is also likely channel mediated (Maathuis and Sanders, 1993). [K⁺]ₜₐ₉ and resting ΔΨₘ values, when measured, are also listed. Asterisks refer to references as follows: *see Maathuis and Sanders (1993), Halperin and Lynch (2003); **see Leigh and Jones (1984), Walker et al. (1996a), Kronzucker et al. (2006).
4.2 Complexity of Potassium Acquisition: How Much Flows Through Channels?

Preface

A modified version of this subchapter was published as: Coskun D and Kronzucker HJ (2013) Complexity of potassium acquisition: How much flows through channels? Plant Signaling & Behavior 8, e24799; PMID: 23656868. This article served as an addendum to the manuscript presented in Subchapter 4.1.

D Coskun wrote the manuscript, with input and editing from HJ Kronzucker.

4.2.1 Abstract

The involvement of potassium ($K^+$)-selective, Shaker-type channels, particularly AKT1, in primary $K^+$ acquisition in roots of higher plants has long been of interest, particularly in the context of low-affinity $K^+$ uptake, at high $K^+$ concentrations, as well as uptake from low-$K^+$ media under ammonium ($NH_4^+$) stress. We recently demonstrated that $K^+$ channels cannot mediate $K^+$ acquisition in roots of intact barley ($Hordeum vulgare$ L.) seedlings at low ($22.5 \, \mu M$) external $K^+$ concentrations ($[K^+]_{ext}$) and in the presence of high (10 mM) external $NH_4^+$, while the model species $Arabidopsis thaliana$ L. utilizes channels under comparable conditions. However, when external $NH_4^+$ was suddenly withdrawn, a thermodynamic shift to passive (channel-mediated) $K^+$ influx was observed in barley and both species demonstrated immediate and dramatic stimulations in $K^+$ influx, illustrating a hitherto unexplored magnitude and rapidity of $K^+$-uptake capacity and plasticity. Here, we expand on our previous work by offering further characterization of channel-mediated $K^+$ fluxes in intact barley, with particular focus on anion effects, root respiration, and pharmacological sensitivity, and highlight key additions to the current model of $K^+$ acquisition.
4.2.2 Main text

The potassium (K⁺)-selective Shaker-type channel, AKT1, has been shown to mediate both high- and low-affinity K⁺ acquisition in roots of higher plants (Hirsch et al. 1998; Cherel 2004; Gierth et al. 2005) and a sophisticated model involving its molecular regulation and relative contribution to K⁺ uptake has emerged, based largely on the work in the model system Arabidopsis (Arabidopsis thaliana L.) (Dennison et al. 2001; Gierth and Mäser 2007; Alemán et al. 2011; Dreyer and Uozumi 2011). According to this view, approximately 80% of high- and low-affinity K⁺ uptake in Arabidopsis can be attributed to the sum of functions of AtHAK5 (a member of the HAK/KUP/KT family of transporters; Mäser et al. 2001) and AtAKT1, while the remaining ~20% is mediated by as yet unidentified components (Gierth and Mäser 2007). Of special interest and the source of some controversy, has been the involvement of AtAKT1 in mediating K⁺ uptake from low (micromolar) external K⁺ concentrations ([K⁺]ₐₗ in the presence of high (millimolar) external ammonium (NH₄⁺) (Hirsch et al. 1998; Spalding et al. 1999). Under such conditions, high-affinity K⁺ uptake is severely suppressed at the functional level, which, in Arabidopsis, has been directly linked to AtHAK5 inhibition (Qi et al. 2008). It has been shown that under such conditions, AtAKT1 can conduct the majority of K⁺ uptake from [K⁺]ₐₗ as low as 10 μM (Hirsch et al. 1998). However, such findings have been difficult to reconcile with thermodynamic considerations that suggest channel-mediated K⁺ acquisition at such low [K⁺]ₐₗ is generally not feasible (Kochian and Lucas 1993; Maathuis and Sanders 1994). Our recent study (Coskun et al. 2013c) showed that the Arabidopsis model of K⁺ acquisition is not a universally applicable one, and that, in particular, it may not apply to cereals. Our work demonstrated that in roots of intact barley (Hordeum vulgare L.) seedlings grown under high (10 mM) NH₄⁺, K⁺ channels could not conduct K⁺ acquisition when [K⁺]ₐₗ was low (22.5 μM), but at intermediate (112.5 μM) [K⁺]ₐₗ, channels operated jointly with high-affinity transporters. At high (5 mM) [K⁺]ₐₗ, K⁺ channels dominated and stimulations of K⁺ influx by anions chloride (Cl⁻) and nitrate (NO₃⁻) were observed. K⁺ efflux was found to be channel-mediated at low and intermediate [K⁺]ₐₗ, albeit with differing pharmacological profiles and no K⁺ efflux was found at high [K⁺]ₐₗ. When external NH₄⁺ was withdrawn, significant stimulations in K⁺ influx were observed (176% increase compared with control), coincident with a thermodynamic shift from active to passive conditions that permitted channel-mediated K⁺ influx even at low [K⁺]ₐₗ. This ammonium-withdrawal effect, termed AWE, was also observed in wild-type and mutant lines of Arabidopsis (athak5, atakt1 and athak5 atakt1). AWE was suppressed by high (1–5 mM) levels
of external calcium (Ca$^{2+}$) at low and intermediate, but not at high, [K$^+$]$_\text{ext}$, which was attributed to Ca$^{2+}$-sensitivity of AKT1. The effect was additionally enhanced by the presence of Cl$^-$ and NO$_3^-$ at high [K$^+$]$_\text{ext}$, conditions under which we observed some of the highest trans-plasma-membrane K$^+$ fluxes ever reported [~36 μmol g$^{-1}$ (root fresh weight) h$^{-1}$]. Furthermore, AWE was sustainable (and at times indeed increasing) over a 24-h period, at low and intermediate [K$^+$]$_\text{ext}$ and persisted for up to ~8 h at high [K$^+$]$_\text{ext}$. This resulted in significant total-tissue K$^+$ accrual at all [K$^+$]$_\text{ext}$ over 24 h. These results raise a suite of interesting questions about the nature of channel-mediated K$^+$ influx in intact plants. Although we showed that K$^+$ channels cannot “do it all,” as suggested by early thermodynamic considerations (Kochian and Lucas 1993), our work sheds light on the wide-ranging and complex operation of K$^+$ channels in planta.

Here, we expand on our previous work by further characterizing channel-mediated K$^+$ uptake in roots of barley. Figure 20 illustrates the varying sensitivity of AWE to Ca(NO$_3$)$_2$, which is clearly a function of [K$^+$]$_\text{ext}$: Ca$^{2+}$-induced suppressions are seen at low and intermediate [K$^+$]$_\text{ext}$ (Fig. 20A and B), while a NO$_3^-$-induced stimulation is seen at high [K$^+$]$_\text{ext}$ (Fig. 20C). Interestingly, under all AWE + Ca(NO$_3$)$_2$ combinations, we observed effective suppression by the K$^+$-channel inhibitors tetraethylammonium (TEA$^+$), barium (Ba$^{2+}$), and cesium (Cs$^+$) (Bertl et al. 1997; Fig. 20A–C). Thus, although Ca$^{2+}$ can block K$^+$ channels (specifically AtAKT1; Coskun et al. 2013c) under some AWE conditions, it appears that K$^+$ channels continue to operate under such suppressed conditions, resulting in a higher flux compared with control (NH$_4^+$-background) conditions (Fig. 20A and B). This is consistent with previous thermodynamic analyses (Coskun et al. 2013c) that showed AWE + Ca(NO$_3$)$_2$ hyperpolarizes root plasma-membrane potentials away from the equilibrium potential for K$^+$ (E$_{K^+}$) at low and intermediate [K$^+$]$_\text{ext}$. At high [K$^+$]$_\text{ext}$, it is evident that NO$_3^-$-induced stimulations in K$^+$ influx are also linked to K$^+$ channels (Fig. 20C), illustrating their complexity of function and regulation (also see below). Interestingly, these elevations in K$^+$ influx compared with control (albeit minor at low and intermediate [K$^+$]$_\text{ext}$) are sufficient to noticeably increase tissue-K$^+$ content over 24 h at all three [K$^+$]$_\text{ext}$ (data not shown), similar to what AWE entails on its own (Coskun et al. 2013c).

Expanding on the anion effects observed at high [K$^+$]$_\text{ext}$, we also found that switching the counter-ions for K$^+$ and NH$_4^+$ from sulfate (SO$_4^{2-}$) to Cl$^-$ (a 10-min treatment was used in SO$_4^{2-}$-grown plants) approximately doubles K$^+$ influx (from ~10 to 20 μmol g$^{-1}$ h$^{-1}$) and the reverse
scenario (switching from Cl\(^-\) to SO\(_4^{2-}\), in Cl\(^-\)-grown plants), reduces influx by half (from ~20 to 10 \(\mu\)mol g\(^{-1}\) h\(^{-1}\), Fig. 20D). This suggests that anion effects related to K\(^+\)-channel functioning are highly effective in both the short (10 min) and long (steady-state) term. These findings are consistent with early reports (Epstein et al. 1963; Kochian et al. 1985) demonstrating higher low-affinity K\(^+\) influx in the presence of Cl\(^-\) over SO\(_4^{2-}\), possibly as a result of a coupling of the more rapidly absorbed anion (Cl\(^-\)) with K\(^+\) influx. Surprisingly, there has been little, if any, advance in understanding this phenomenon. It does, however, raise interesting questions about the mechanism and regulation of channel-mediated K\(^+\) influx. For one, when one considers that the fluxes of all anions tested (Cl\(^-\), SO\(_4^{2-}\), and NO\(_3^-\)) are coupled to H\(^+\) influx and are electrogenic (net positive; Sanders 1980; Ullrich and Novacky 1981; Lass and Ullrich-Eberius 1984), anion fluxes (particularly those of NO\(_3^-\) and Cl\(^-\), which show high rates of uptake; Lee 1982) should theoretically decrease the gradient for K\(^+\) uptake, i.e. working against what is observed for rates of influx (Fig. 20C and D; Epstein et al. 1963; Kochian et al. 1985). Moreover, to our knowledge, there are no demonstrations of possible allosteric modulations of Shaker-type K\(^+\) channels by anions in the plant literature (Gambale and Uozumi 2006; Dreyer and Uozumi 2011); by contrast, some evidence exists in the animal literature (Adams and Oxford 1983) that such allosteric modulation may occur. This warrants further investigation. Lastly, the reasons why Cl\(^-\)/NO\(_3^-\)-induced stimulations are only observed at high (low-affinity-range) [K\(^+\)]\(_{\text{ext}}\) remain largely unknown. Reports on the allosteric modulation of Shaker-type K\(^+\) channels by [K\(^+\)]\(_{\text{ext}}\) appear exclusive to outward rectifiers (Johansson et al. 2006), and studies on the post-translational modification (e.g., phosphorylation/dephosphorylation networks) of inward rectifiers are restricted to low-[K\(^+\)]\(_{\text{ext}}\) sensing (Li et al. 2006; Xu et al. 2006; Lee et al. 2007). Thus, it is clear that as yet insufficiently understood mechanisms exist regarding the regulation of K\(^+\) channels by anions, a phenomenon that can have profound effects on rates of K\(^+\) acquisition.
Figure 20. K⁺ influx into intact 7-d old barley (Hordeum vulgare L.) seedlings. (A–C) Steady-state (control) flux in plants grown in a full nutrient medium (1/4-strength Johnson’s solution) with 10 mM NH₄⁺ and either 0.0225 (A), 0.1125 (B) or 5 mM (C) K⁺ (as sulfate salts) and the effect of ammonium withdrawal (AWE) ± Ca(NO₃)₂ (5 mM) and AWE + Ca(NO₃)₂ ± TEA⁺ (10 mM), Ba²⁺ (5 mM), or Cs⁺ (10 mM) (as chloride salts). All treatments involved 5-min incubation prior to ⁴²K⁺ uptake (5-min protocol). (D) Influx into plants grown in a full-nutrient medium (as above) with 5 mM K⁺ and 10 mM NH₄⁺ (both as either SO₄²⁻ or Cl⁻ salts) and the effect of switching (K⁺ and NH₄⁺) counter-ions (10-min incubation prior to uptake, as above). Influx measured on a per g (root fresh weight) basis. Error bars indicate ± SEM of minimum four replicates. Letters in each panel denote significantly different means (P < 0.05, one-way ANOVA with Tukey post-hoc test).
We also observed that NH$_4^+$ withdrawal (for 5 min) results in significant reductions in root respiration at low [K$^+$]$_{ext}$ (from ~30 to 20 μmol g$^{-1}$ h$^{-1}$, as measured by oxygen (O$_2$) consumption; Fig. 21). Based on earlier suggestions, this may be attributable to a reduction in futile NH$_4^+$ cycling at root-cell plasma membranes, an energetically demanding scenario linked to NH$_4^+$ toxicity in barley (Britto et al. 2001b), or other, more generic respiratory stress responses when NH$_4^+$ levels are high. Since futile cycling of NH$_4^+$ can be reduced by high [K$^+$]$_{ext}$ (Szczerba et al. 2008b), as evident in the significantly lower steady-state root respiration at high [K$^+$]$_{ext}$ (Fig. 21), this may explain the lack of any further respiratory drop upon NH$_4^+$ withdrawal. Given the substantial increases in K$^+$ influx (from ~10 to 25 μmol g$^{-1}$ h$^{-1}$) (Fig. 21C), it is interesting that no change in root respiration was observed, in keeping with the contention that the energy cost per K$^+$ transported is significantly lower for channels than for high-affinity transporters (Glass, 1989), i.e., plants already engaging channels may show no further significant energetic requirement upon NH$_4^+$ withdrawal even when K$^+$ influx is greatly enhanced. Thus, AWE carries no major cost to the plant, while a significant benefit in terms of net K$^+$ accumulation in tissue is seen (see above). Indeed, at low [K$^+$]$_{ext}$, barley expends significantly less energy than prior to NH$_4^+$ withdrawal, while accruing large quantities of K$^+$. This highlights an important feature of engaging channels episodically in the acquisition of potassium that may be of importance under fluctuating nutrient conditions (Hodge 2004), especially in NH$_4^+$-dominated systems that normally suppress K$^+$ uptake (Sasakawa and Yamamoto 1978; Kronzucker et al. 1997; Kirk and Kronzucker 2005), which, integrated over an extended time frame, may result in significant growth benefits (Balkos et al. 2010).
Figure 21. Steady-state (control) root O$_2$ consumption, as measured using a Hansatech oxygen electrode and Oxygraph control system (Hansatech Intruments), in intact barley (*Hordeum vulgare* L.) seedlings grown in a full-nutrient medium at 0.0225 or 5 mM K$^+$ and 10 mM NH$_4^+$ (both as sulfate salts) and the effect of sudden (5-min treatment) NH$_4^+$ withdrawal (AWE). O$_2$ flux measured on a per g (root fresh weight) basis. Error bars indicate ± SEM of 4 replicates. Letters denote significantly different means (P < 0.05, one-way ANOVA with Tukey post-hoc test).
4.3 How High Do Ion Fluxes Go? A Re-Evaluation of the Two-Mechanism Model of K⁺ Transport in Plant Roots

Preface

A modified version of this subchapter was published as: Coskun D, Britto DT, Kochian LV, Kronzucker HJ (2016) How high do ion fluxes go? A re-evaluation of the two-mechanism model of K⁺ transport in plant roots. *Plant Science* **243**, 96-104. Supplementary material cited in this subchapter can be found online:

HJ Kronzucker designed the study, with input from DT Britto and D Coskun. D Coskun performed the experiments and analyzed the data, with input from DT Britto and HJ Kronzucker. D Coskun wrote the manuscript, with input and editing from DT Britto, LV Kochian, and HJ Kronzucker.

4.3.1 Abstract

Potassium (K⁺) acquisition in roots is generally described by a two-mechanism model, consisting of a saturable, high-affinity transport system (HATS) operating via H⁺/K⁺ symport at low (<1 mM) [K⁺]_{ext}, and a linear, low-affinity system (LATS) operating via ion channels at high (>1 mM) [K⁺]_{ext}. Radiotracer measurements in the LATS range indicate that the linear rise in influx continues well beyond nutritionally relevant concentrations (>10 mM), suggesting K⁺ transport may be pushed to extraordinary, and seemingly limitless, capacity. Here, we assess this rise, asking whether LATS measurements faithfully report transmembrane fluxes. Using ⁴²K⁺-isotope and electrophysiological methods in barley, we show that this flux is part of a K⁺-transport cycle through the apoplast, and masks a genuine plasma-membrane influx that displays Michaelis–Menten kinetics. Rapid apoplastic cycling of K⁺ is corroborated by an absence of transmembrane ⁴²K⁺ efflux above 1 mM, and by the efflux kinetics of PTS, an apoplastic tracer. A linear apoplastic influx, masking a saturating transmembrane influx, was also found in Arabidopsis mutants lacking the K⁺ transporters AtHAK5 and AtAKT1. Our work significantly revises the model of K⁺ transport by demonstrating a surprisingly modest upper limit for plasma-membrane influx, and offers insight into sodium transport under salt stress.
4.3.2 Introduction

The two-mechanism model of potassium (K+) acquisition in roots of higher plants is a standard of transport physiology and molecular biology (Epstein et al. 1963; Glass and Dunlop 1978; Kochian and Lucas 1982; Gierth and Mäser 2007; Szczerska et al. 2009). In this model (Fig. 22A), the high-affinity transport system (HATS) predominately functions at external K+ concentrations ([K+]_ext) below 1 mM, and is mediated by secondarily active K+/H+ symporters of the HAK/KUP/KT family, including AtHAK5 in Arabidopsis (Arabidopsis thaliana; Gierth and Mäser 2007) and HvHAK1 in barley (Hordeum vulgare; Santa-Maria et al. 1997). By contrast, the low-affinity transport system (LATS), which functions at [K+]_ext above 1 mM, is less well understood, although it is widely accepted to be a thermodynamically passive process mediated by K+-selective (Shaker-like) channels (e.g. AtAKT1 and HvAKT1 in Arabidopsis and barley, respectively; Kochian and Lucas 1982; Gassmann and Schroeder 1994; Gierth and Mäser 2007; Szczerska et al. 2009). Studies using heterologous expression systems and knock-out mutants in Arabidopsis have furthermore shown that AtAKT1 can also operate under some high-affinity conditions (Hirsch et al. 1998; Geiger et al. 2009; Honsbein et al. 2009; Coskun et al. 2014b). Moreover, recent investigations in the Arabidopsis double-knock-out mutant, athak5 atakt1, have shown that there is an additional back-up system (“BUS”) that can contribute to K+ uptake in the LATS range (Pyo et al. 2010; Caballero et al. 2012; Coskun et al. 2013c). Although genetically uncharacterized, it has been suggested that BUS operates via non-selective cation channels (NSCCs), which may be gated by cyclic nucleotides (Caballero et al. 2012).

One commonly observed feature of the K+ LATS is its linearly rising kinetic response to increasing [K+]_ext, which contrasts sharply with the saturating pattern typical of the HATS (Fisher et al. 1970; Robinson and Laties 1975; Polley and Hopkins 1979; Kochian et al. 1985; Vallejo et al. 2005; see also Fig. 22). From the outset, it is worth noting that this model is based solely on unidirectional fluxes measured using radiotracer methodology, and although many aspects have been corroborated by other experimental methods, the linearity of the low-affinity flux is one aspect that has not. Any attempt to investigate this important concept must therefore also employ radiotracer techniques. Moreover, although many studies have reported on a linear LATS, it is important to note that some studies have reported low-affinity K+ influxes that saturate (Epstein et al. 1963; Maathuis and Sanders 1996a; Gierth et al. 2005). It is interesting to note that such studies often employ longer absorption and desorption times (e.g. 10-30 min...
each), compared to many studies that report linear fluxes, which may result in closer approximations to net $K^+$ fluxes, rather than unidirectional uptake (see below). Furthermore, a linear LATS is not unique to $K^+$, but has been observed with many ions, including sodium ($Na^+$), ammonium ($NH_4^+$), chloride ($Cl^-$), and nitrate ($NO_3^-$) (Ullrich et al. 1984; Kochian et al. 1985; Glass et al. 1992; Wang et al. 1993b; Essah et al. 2003), as well as with organic solutes such as amino acids and sugars (Blackman and McDaniel 1978; Fischer and Luttge 1980; Lichtner and Spanswick 1981). Such fluxes are generally considered to be channel-mediated, and typically show no signs of saturation at external substrate concentrations between 10 and 50 mM. Only a few studies have examined fluxes beyond this range because such conditions are often considered physiologically or ecologically irrelevant, and, moreover, potentially damaging to cells. An important exception is that of $Na^+$ fluxes in the context of salinity stress, where the measurement of fluxes at very high substrate concentrations (e.g. >100 mM) is both appropriate and routine. In this case, extraordinarily high fluxes are frequently reported, and although believed to be mediated by NSCCs, their mechanistic underpinnings remain poorly defined (Kronzucker and Britto 2011). By contrast, much more is known about $K^+$ transport into roots and within the plant (Szczerba et al. 2009; Ahmad and Maathuis 2014; Nieves-Cordones et al. 2014); thus, we are better equipped to investigate the nature of the $K^+$ LATS, and use results from this model system to gain insight into $Na^+$ transport at high external concentrations.

Another critical aspect of low-affinity transport in general involves the efflux of ions, which occurs simultaneously with influx, and appears to rise more steeply than influx does, as external concentrations increase (Britto and Kronzucker 2006). Such patterns have been observed for all major nutrient ions, including $K^+$, $Na^+$, $Cl^-$, $NO_3^-$, $NH_4^+$, and sulfate ($SO_4^{2-}$), and efflux in the low-affinity range frequently achieves rates approximating those of influx (i.e. efflux:influx ratios become close to unity). This futile cycling of ions typically carries with it a potentially substantial energetic burden on root systems, given that efflux or influx components of the cycle may occur against significant thermodynamic gradients (Essah et al. 2003; Britto and Kronzucker 2006; Kurimoto et al. 2004).

High rates of efflux make the accurate measurement of low-affinity unidirectional influx, by use of tracer methods, a challenging prospect (Britto and Kronzucker 2001). In order to limit the effect of efflux occurring during tracer-influx measurement protocols, many researchers advocate the use of very short labeling times (e.g. 2–5 min; Essah et al. 2003; Malagoli et al.
2008; Wang et al. 2009). For the same reason, short desorption times (i.e. “washes” in non-labeled solutions following uptake, to remove extracellular tracer; see Chapter 7) are often prescribed. Although such protocols have become standard in the measurement of low-affinity fluxes (e.g. see Kronzucker and Britto 2011), the assumptions that they minimize tracer efflux across the plasma membrane, and clear the roots of extracellular tracer, are rarely tested, and indeed may be untenable in some cases. For example, we recently showed that K⁺ efflux across the plasma membrane in roots of intact barley seedlings ceases above a [K⁺]ₜₐₚ of 1 mM (i.e. in the low-affinity range), and concluded that tracer release from pre-labeled roots was extracellular (apoplastic) in origin (Coskun et al. 2010; see also Subchapter 3.1). The implications from this study for low-affinity influx measurements require further exploration.

Here, we ask three fundamental questions regarding the nature of low-affinity K⁺ influx in roots of higher plants: (1) Does influx have an upper limit and eventually saturate with rising [K⁺]ₜₑₓᵗ, or continue to rise indefinitely? (2) What mechanism(s) underlie its linear concentration dependence? (3) Can a better understanding of low-affinity K⁺ transport help resolve the mechanisms of Na⁺ fluxes under salt stress? We shall argue that transmembrane fluxes do not rise indefinitely, but saturate at relatively modest rates (i.e. 7–15 µmol g⁻¹ root fresh weight (FW) h⁻¹), well into the saline range (≤100 mM [K⁺]ₜₑₓᵗ), while the linearly rising component of the measured flux is dominated by the apoplastic movement of K⁺. We believe that our findings call for a fundamental revision of flux models, and apply to low-affinity fluxes of other ions, particularly those of Na⁺.
Figure 22. The two-mechanism model of K⁺ acquisition in roots of higher plants and its extension into the saline range. (A) The standing model of K⁺ transport. Influx (solid black line), as a function of external [K⁺] ([K⁺]_{ext}), is described as the sum of activities of two distinct transport systems: (I) the saturable high-affinity transport system (HATS; blue dashed line), which follows Michaelis–Menten kinetics and is regulated by internal K⁺ status; and (II) the low-affinity transport system (LATS; red dotted line), which is linear. The HATS is primarily governed by HAK/KUP/KT transporters, whereas the LATS is governed by AKT1-channel complexes and unknown back-up system(s). Here we ask whether influx will continue to rise linearly beyond nutritionally relevant [K⁺]_{ext} (i.e. >10 mM, and into the saline range). Redrawn from Kochian and Lucas (1982); Britto and Kronzucker (2008). (B) K⁺ influx into roots of intact barley seedlings, as a function of [K⁺]_{ext}, and its dependence on desorption time (5 min, blue circles; 30 min, red triangles). Seedlings were grown on full-nutrient media supplemented with 0.1 mM K⁺. Labeling time = 5 min. (C) Influx as a function of labeling time, in barley seedlings grown and measured under 100 mM K⁺. Desorption time = 5 min. Throughout, error bars represent ± SEM (n ≥ 4).
4.3.3 Results

Low-Affinity $K^+$ Influx Values are Highly Dependent on Protocol and Show no Sign of Saturation

To ascertain the upper limits of the $K^+$ LATS, we used $^{42}K^+$ to measure $K^+$ influx in intact seedlings of barley between 10 and 100 mM $[K^+]_{\text{ext}}$. We also explored the effect that timing protocols (i.e. labeling and desorption times) had on influx estimation, to assess the recommendations that have been put forth in the literature (see Section 4.3.2). Fig. 22B shows the response of $K^+$ influx with a fixed labeling time of 5 min and either a 5- or a 30-min desorption protocol. We found that apparent influx was significantly ($P < 0.001$) higher with the shorter desorption times throughout the range tested, as was the slope of the isotherms (Table S1). Nevertheless, with both desorption times, we observed a strong ($R^2 = 0.90$) linear response of influx to $[K^+]_{\text{ext}}$, with no indication of saturation. In addition, using a 5-min labeling, 5-min desorption protocol, we measured influx at extremely high concentrations of $K^+$ (up to two molar), but still observed a linear response (Fig. S1). In steady-state experiments conducted at 100 mM $[K^+]_{\text{ext}}$, we fixed the desorption time at 5 min, while varying the labeling time between 1 and 60 min. We observed a strong decline in the flux, from 90 to 45 $\mu$mol g$^{-1}$ h$^{-1}$, as labeling time increased between 1 and 10 min. After 30–60 min of desorption, the flux value did not change further, settling at about 20 $\mu$mol g$^{-1}$ h$^{-1}$ (Fig. 22C).

Cellular $K^+$ Efflux Ceases in the Low-Affinity Range and Apoplastic Fluxes Dominate

Because declines in influx with increasing duration of labeling and desorption times are usually attributed to simultaneous tracer efflux (Britto and Kronzucker 2001; Essah et al. 2003), the efflux step in the LATS range was examined more closely. Fig. 23A illustrates the strong malleability in response to various chemical treatments, of $^{42}K^+$ efflux from roots of intact, labeled barley seedlings grown and measured under high-affinity conditions (0.1 mM $[K^+]_{\text{ext}}$). The $K^+$-transport inhibitor cesium ($Cs^+$) strongly and immediately suppressed $^{42}K^+$ efflux, as did elevated levels of externally supplied $K^+$ (10 mM), whereas the metabolic inhibitor cyanide (CN$^-$) significantly stimulated efflux. By contrast, similar treatments had no effect on efflux traces from roots of plants grown and measured under low-affinity conditions (1 and 40 mM.
[K\textsuperscript{+}]_{\text{ext}}; Fig. 23B and C, respectively). In addition, an alkaline (pH 9.2) treatment, designed to collapse the H\textsuperscript{+} gradient across the plasma membrane, and thus disable possible H\textsuperscript{+}-K\textsuperscript{+} exchangers (Pardo et al. 2006), had no effect on efflux at 1 and 40 mM [K\textsuperscript{+}]_{\text{ext}}. To further test the hypothesis that \textsuperscript{42}K\textsuperscript{+} efflux traces under LATS conditions could be apoplastic in origin, we monitored the release kinetics of the apoplastic dye 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS; Yeo et al. 1987) from PTS-loaded roots, and observed release patterns that were very similar to those of \textsuperscript{42}K\textsuperscript{+} (Fig. 23D). Importantly, the half-time for PTS release from the root apoplast (t\textsubscript{1/2} = 12.8 ± 1.4 min) fell precisely into the range of half-times for K\textsuperscript{+} release in the low-affinity range (t\textsubscript{1/2} = 10–18 min; Table 9).
Figure 23. Cellular K⁺ efflux ceases in the low-affinity range and what is measured is extracellular (apoplastic) in origin. (A) $^{42}$K⁺ efflux from roots of intact barley seedlings grown and measured at 0.1 mM K⁺, and the response to the sudden (at $t = 16$ min; see arrow) exposure of either 10 mM Cs⁺ (red squares), 1 mM CN⁻ (green triangles), or high (10 mM) K⁺ (orange diamonds). (B and C) As in panel A, but with plants grown and measured at 1 and 40 mM K⁺, respectively, and exposed to alkalinity (pH 9.2; purple crosses). (D) PTS release from pre-labeled (0.1% PTS (w/v)) roots of intact barley seedlings grown as in panel A. Under all conditions, compartmental analysis of the slowly-exchanging phase (dotted line) was conducted (see Table 9 for extracted parameters). Each point represents the mean of a minimum 3 replicates (SEM < 15% of the mean). (A and B) Redrawn from Coskun et al. (2010).
Table 9. Compartmental analysis in roots of intact barley (*Hordeum vulgare*) seedlings grown and measured with full-nutrient media supplemented with 0.1, 1, or 40 mM K\(^+\). Release kinetics of the apoplastic dye PTS from pre-labeled (0.1% PTS (w/v)) roots were also monitored from seedlings grown on 0.1 mM K\(^+\).

<table>
<thead>
<tr>
<th>[K(^+)](_{ext})</th>
<th>Influx (I)</th>
<th>Efflux (E)</th>
<th>Net flux</th>
<th>E:I Ratio</th>
<th>Half-time (t(_{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>µmol g(^{-1}) (root FW) h(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td>min</td>
</tr>
<tr>
<td>0.1</td>
<td>7.22 ± 0.23</td>
<td>1.86 ± 0.18</td>
<td>5.36 ± 0.18</td>
<td>0.25 ± 0.02</td>
<td>28.18 ± 3.40</td>
</tr>
<tr>
<td>1*</td>
<td>5.97 ± 0.77</td>
<td>0.57 ± 0.03</td>
<td>5.40 ± 0.77</td>
<td>0.11 ± 0.01</td>
<td>17.56 ± 1.36</td>
</tr>
<tr>
<td>40*</td>
<td>32.52 ± 2.88</td>
<td>23.91 ± 2.30</td>
<td>8.61 ± 0.71</td>
<td>0.73 ± 0.01</td>
<td>9.88 ± 1.20</td>
</tr>
<tr>
<td>PTS*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.8 ± 1.4</td>
</tr>
</tbody>
</table>

* Measurements from the apoplast.
**Transmembrane K⁺ Influx Saturates in the Low-Affinity Range**

We hypothesized that the linear response of influx to rising \([K^+]_{\text{ext}}\) (Fig. 22B) was the result of apoplastic tracer retention even after substantial desorption. To test this, we monitored the sensitivity of the flux to a cocktail of \(K^+\)-channel blockers, \(Cs^+\) (10 mM) and tetraethylammonium (TEA⁺; 10 mM), along with low temperature (4 °C), in order to suppress overall metabolic and transport activity (Bravo and Uribe 1981). In plants grown at 0.1 mM \(K^+\) in a full-nutrient medium, influx was significantly (\(P < 0.05\)) inhibited by these treatments throughout the range tested (10–100 mM \([K^+]_{\text{ext}}\)), but nevertheless the component of apparent \(K^+\) influx insensitive to inhibition rose linearly with increasing \([K^+]_{\text{ext}}\) (Fig. 24B). Nevertheless, the fraction of the control flux amenable to inhibition remained surprisingly constant, near 10 µmol g⁻¹ h⁻¹ (Fig. 24B).

When we applied these treatments to plants that were either nutrient-deprived (grown on 200 µM \(CaSO_4\); Fig. 24A) or \(K^+\)-replete (grown on a full-nutrient medium with 10 mM \(K^+\); Fig. 24C), we observed very similar responses in influx (i.e. linear responses to \([K^+]_{\text{ext}}\) and a fairly uniform inhibition throughout the tested range). However, the flux amenable to inhibition was inversely proportional to \(K^+\) supply: in \(K^+\)-starved plants, it was \(\sim 12\) µmol g⁻¹ h⁻¹ (Fig. 24A), whereas, in \(K^+\)-replete plants, it was \(\sim 7\) µmol g⁻¹ h⁻¹ (Fig. 24C). In both cases, this malleable flux remained relatively constant, i.e. it saturated throughout the \(K^+\) concentration range tested.

Subsequently, we tested Arabidopsis double-knock-out mutants lacking AtHAK5 and AtAKT1, the two principal \(K^+\) transporters involved in \(K^+\) uptake by roots (Caballero et al. 2012; Coskun et al. 2013c). When we applied a cocktail of the inhibitors \(Cs^+\), TEA⁺, and calcium (\(Ca^{2+}\), at 15 mM, a concentration at which \(Ca^{2+}\) inhibits the third “back-up system”, BUS; Caballero et al. 2012), we observed nearly uniform inhibition of influx across the tested concentration range, with saturation near 13 µmol g⁻¹ h⁻¹ (Fig. 24D). Importantly, in the presence of both a double genetic knock-out of \(K^+\) transport, and a stacked transport inhibitory treatment, we nevertheless still observed a continuous linear rise of \(K^+\) influx into plant roots (Fig. 24D).

Lastly, electrophysiological analysis was applied to \(K^+\) transport in roots of barley seedlings, which showed a saturating depolarization in root epidermal and cortical cells over the same range of \([K^+]_{\text{ext}}\) (10–100 mM; Fig. 25). In plants grown and treated as in Fig. 24B, root plasma-membrane potentials depolarized rapidly upon exposure to 10 mM \([K^+]_{\text{ext}}\) and remained
relatively constant, *i.e.* saturated, up to 100 mM. Michaelis–Menten analysis of the electrical response indicated a half-saturation constant (K_M) of 2.5 mM.
Figure 24. Trans-membrane K⁺ influx saturates in the low-affinity range, despite an apparent linear isotherm, and is inversely proportional to plant K⁺ status. (A) Apparent K⁺ influx in roots of intact barley seedlings between 20–100 mM [K⁺]_{ext}, and the effect of Cs⁺ (10 mM) + TEA⁺ (10 mM) at 4 °C. Seedlings were grown under nutrient-deprived conditions (200 µM CaSO₄ solution). Labeling time = 5 min; desorption time = 30 min. Error bars represent ± SEM (n ≥ 4). (B and C) As in panel A, but in plants grown under full-nutrient media supplemented with 0.1 or 10 mM K⁺, respectively. (D) Apparent K⁺ influx with and without the presence of Cs⁺ (10 mM) + TEA⁺ (10 mM) + Ca²⁺ (15 mM), measured in roots of intact athak5 atakt1 Arabidopsis seedlings. Seedlings were grown on a full-nutrient media supplemented with 1.5 mM K⁺ for 4 weeks, followed by one week on 0.1 mM K⁺. Labeling time = 5 min; desorption time = 5 min. Throughout, filled circles represent control fluxes, open circles represent inhibitor treatments, and red triangles represent transmembrane influx, determined as the arithmetic difference between control and treatment pairs. Error bars represent ± SEM (n ≥ 3).
Figure 25. Plasma-membrane depolarization saturates with rising $[K^+]_{\text{ext}}$. Depolarization ($\Delta \Delta \Psi_m$) in response to rising $[K^+]_{\text{ext}}$ in root epidermal and cortical cells of intact barley seedlings grown under a full-nutrient medium supplemented with 0.1 mM $K^+$. Data is fitted to a Michaelis–Menten regression analysis ($K_M = 2.46$ mM, $V_{\text{max}} = 66.2$ mV). Resting membrane potential ($\Delta \Psi_m$) = $-155.6 \pm 10.7$ mV. Error bars represent ± SEM (n ≥ 3).
4.3.4 Discussion

This study critically re-examines the standing two-mechanism model of $K^+$ transport in roots, with specific focus on the nature of the linear low-affinity system. As previously reasoned and demonstrated (see Schroeder and Fang 1991, Maathuis and Sanders 1994, Scherzer et al. 2015, in the case of $K^+$; Davenport and Tester 2000, Mian et al. 2011, in the case of $Na^+$; and Coronado et al. 1980, Blatz and Magleby 1984, Hille 2001, for examples from the animal literature), even a channel-mediated flux (as measured by whole-cell and patch-clamp electrophysiology) must eventually saturate with sufficiently high substrate concentration, but we nevertheless observed the classic linear response of the LATS when measured using classical radiotracer techniques, which continued even at very high substrate concentrations, up to 2 M $[K^+]_{\text{ext}}$ (Fig. S1). Clearly, fluxes at 2 M $[K^+]_{\text{ext}}$ cannot be attributable to transmembrane transport events, not only because they are energetically unfeasible (see below), but also because membrane disruption generally occurs at far lower concentrations (Britto et al. 2010). Thus, at the maximal concentrations tested, a non-physiological, extracellular, component is almost certainly responsible for the continued linear rise in $K^+$ uptake. This raises a more significant question: to what extent does the apparent flux represent genuine transmembrane transport of $K^+$ at more moderate low-affinity concentrations (e.g. 10–100 mM; Figs. 22 and 24)?

To address this question, we next investigated the nature of $K^+$ efflux under low-affinity conditions. In our present and previous (Coskun et al. 2010; Subchapter 3.1) analyses, we found no evidence for transmembrane efflux from barley roots to the external solution in the LATS range, under both steady-state and non-steady-state conditions, and at the low (1 mM) and higher (40 mM) end of the LATS range (Fig. 23; see also Coskun et al. 2010; Subchapter 3.1). By contrast, transmembrane $^{42}K^+$ efflux was readily demonstrable in the HATS range (0.1 mM; Fig. 23A). Under non-steady-state conditions, a switch from 0.1 to 10 mM $K^+$ resulted in an immediate suppression of $K^+$ efflux to the same extent as produced by the application of $Cs^+$ (Fig. 23A), suggesting that efflux had been effectively silenced by the switch to a higher external $K^+$ concentration alone. This was further supported by the observation that efflux was equally suppressed by higher (50 mM) concentrations of $K^+$ (not shown). The effect of increased $[K^+]_{\text{ext}}$ on efflux provides strong in planta support for molecular characterizations of outward-rectifying $K^+$ (KOR) channels that are gated shut at elevated $[K^+]_{\text{ext}}$ (Johansson et al. 2006). Thus, as previously hypothesized (Coskun et al. 2010), tracer efflux from roots under low-affinity
conditions may be simply extracellular (apoplastic) in nature. A similar hypothesis has been put forward in the case of Na\(^+\) efflux under saline conditions, based on an extensive literature review (Britto and Kronzucker 2015). In the case of K\(^+\), this interpretation is buttressed by the similarity in release kinetics of the apoplastic tracer PTS \(t_{1/2} = 12.8 \pm 1.4\) min; Table 9) and that of low-affinity K\(^+\) release \(t_{1/2} = 10–18\) min; Table 9). In agreement with our findings, Yeo et al. (1987) showed that PTS release from intact rice roots can display exchange kinetics in this range \(t_{1/2} \approx 22\) min). It is particularly important to note, however, that with *bona fide* transmembrane K\(^+\) fluxes, such as under high-affinity conditions (Fig. 23A), turnover rates (half-times of exchange) of the cytosolic K\(^+\) pool can also be very similar to those of apoplastic PTS and (putatively apoplastic) K\(^+\) pools discussed here (e.g. at 0.1 mM [K\(^+\)]\(_{\text{ext}}\), \(t_{1/2} = 28.18 \pm 3.40\) min; Table 9), rendering the distinction between symplastic and apoplastic fluxes highly challenging.

Because of the high efflux:influx ratios observed at high [K\(^+\)]\(_{\text{ext}}\) (Britto and Kronzucker 2006; see also Table 9), and the strong possibility that efflux under such conditions does not proceed across the plasma membrane, it is likely that unidirectional influx measurements are substantially overestimated due to the presence of a slow component of apoplastic K\(^+\) flux. Although longer (30-min) desorption protocols can be useful for removing tracer from the apoplast (Fig. 22B), it is likely that this clearance is still not complete, given the relatively long half-times of this phase of the apoplast \(t_{1/2} = 10–18\) min), and thus it is important to differentiate between transmembrane and apoplastic influxes even after long desorption times. We therefore tested the effect of a range of inhibitory treatments (TEA\(^+\), Cs\(^+\), NH\(_4\)^+, CN\(^-\) + SHAM, and 4 °C, separately, and in combination; not shown), the most efficacious of which was a combination of Cs\(^+\) and TEA\(^+\) at 4 °C. Although influx was significantly suppressed using this inhibitory cocktail, we nevertheless observed a residual, non-malleable K\(^+\) flux that maintained the strong linear response to rising [K\(^+\)]\(_{\text{ext}}\) (Fig. 24), typical of LATS transport in the current model (Fig. 22A). Interestingly, similar observations of decreasing malleability of low-affinity fluxes with rising substrate supply were made in several earlier ion-transport studies. For example, Barber (1972) showed that the extent of inhibition of Rb\(^+\) and PO\(_4^3\)- influx, due to cold temperature (0.2 °C) or the metabolic inhibitor 2–4 dinitrophenol, decreased with rising substrate supply. Similarly, Epstein and Hagen (1952) showed that Na\(^+\) uptake under hypoxia (N\(_2\) bubbling), although highly suppressed by the treatment, nevertheless rose significantly with increased substrate supply. Polley and Hopkins (1979) showed that \(^{86}\)Rb\(^+\) influx increased linearly in the presence of the strong uncoupler
CCCP, at substrate concentrations up to 10 mM. Lastly, Kochian et al. (1985) observed a persistent linear $K^+$ ($^{86}\text{Rb}^+$) influx in the presence of TEA+, up to 10 mM Rb+ in excised corn roots. Although it is important to note that the efficacy of inhibitors will likely not be perfect (especially in the case of competitive inhibitors where substrate (e.g. K+) supply can be relatively high), such findings may be interpreted as resulting from an apoplastic flux that is unaffected by metabolic inhibitors, and which increases with rising substrate supply.

Indeed, it has been assumed that the LATS for various ions are relatively less metabolically sensitive than their HATS counterparts, as indicated by $Q_{10}$ analyses (Barber 1972; Polley and Hopkins 1979; Glass et al. 1992; Wang et al. 1993b), even though they are characterized by much higher fluxes (Szczerba et al. 2009; see also Figs. 22, 24, and Fig. S1), which (if they are truly transmembrane fluxes) must ultimately depend on active, and energy-intensive, rectification of thermodynamic gradients by the plasma-membrane H+ -ATPase and other mechanisms (Britto and Kronzucker 2009). Fig. S2 shows the estimated respiratory requirement (in terms of root $O_2$ consumption) for low-affinity K+ influxes in barley seedlings, using established models for ion-$O_2$ flux relationships (Kurimoto et al. 2004; Britto and Kronzucker 2009). In this model, depolarization of the membrane electrical potential due to the thermodynamically passive, uniseriate uptake of K+ via ion channels (i.e. Shaker-like AKT1 complexes and possibly NSCCs, in the case of the “back-up system”; Doyle et al. 1998; Caballero et al. 2012) is compensated for by the active extrusion of protons via the H+ -ATPase (a 1 K+:1 H+ stoichiometry). Secondly, for every 1 H+ pumped via the H+ -ATPase, 1 ATP molecule is consumed via hydrolysis (Palmgren 2001), and thirdly, a stoichiometry of 5 ATP molecules synthesized for every $O_2$ molecule consumed in respiration is observed (i.e. the ‘phosphorylation efficiency’ or P:$O_2$ ratio; Kurimoto et al. 2004; Britto and Kronzucker 2009). In sum, the depolarization brought about by five K+ ions entering the cell passively, via channels, is compensated for energetically with one $O_2$ consumed in respiration. As shown in Fig. S2A, the respiratory $O_2$ demand for K+ influx ≤100 mM $[K^+]_{\text{ext}}$ is at most ~8 $\mu$mol $O_2$ g$^{-1}$ root FW h$^{-1}$, roughly half the total $O_2$ flux measured directly in roots of intact seedlings. Although still feasible at these levels, the energetic demands are pronounced, and become very problematic in the case of K+ fluxes measured above 100 mM $[K^+]_{\text{ext}}$. By 200 mM $[K^+]_{\text{ext}}$, the $O_2$ flux required to compensate for a K+ influx of >100 $\mu$mol g$^{-1}$ h$^{-1}$ exceeds the total energy supply of the plant (Fig. S2B), leaving no room for other respiratory demands (e.g. growth, maintenance, and the
fluxes of other substrates). Such energetic inconsistencies were initially discovered in the case of Na\(^+\) fluxes under salt stress, and call into question the plausibility of extremely high transmembrane ion fluxes, as have been seen with sodium (Britto and Kronzucker 2009), and here in the case of K\(^+\). The energy demands would become even greater were one to postulate a concomitantly operating active-efflux mechanism, such as a K\(^+\)/H\(^+\) antiporter (e.g. via CHX proteins; Pardo et al. 2006), at the plasma membrane (Fig. S2; Table S2; however, see above).

Taken together, our findings strongly suggest that K\(^+\)-influx traces, as obtained by established protocols, are unlikely to accurately represent genuine transmembrane K\(^+\) fluxes. However, a genuine membrane flux may still be estimated by subtracting the non-malleable fluxes (representing apoplastic fluxes; Fig. 24) from the uninhibited control fluxes. Interestingly, this subtraction exercise reveals a possible ‘transmembrane’ flux that follows Michaelis–Menten kinetics, and is saturated between 10 and 100 mM [K\(^+\)]\text{ext}. This flux is therefore not compatible with the paradigm of an indefinitely linearly rising transmembrane influx, but exhibits a concentration-dependence pattern remarkably similar to those found in enzyme kinetics and high-affinity transport systems. Corroborating evidence for a saturable transmembrane influx is provided by electrophysiological experiments showing a saturable depolarization response in root epidermal and cortical cells in intact barley seedlings exposed to 10–100 mM [K\(^+\)]\text{ext} (Fig. 25). Michaelis-Menten analysis of the electrical response yields an apparent K\(_M\) of 2.5 mM, in good agreement with reported kinetic values for channel-mediated fluxes (Epstein et al. 1963; Schroeder and Fang 1991; Gassmann and Schroeder 1994; Maathuis and Sanders 1995; 1996a; Gierth et al. 2005). K\(_M\) values from tracer-uptake experiments were not obtained since transmembrane K\(^+\) influx had already appeared to reach its maximal rate (V\(_{\text{max}}\)) by 10 mM [K\(^+\)]\text{ext} (Fig. 24); this apparent V\(_{\text{max}}\) ranged from 7 to 12 μmol g\(^{-1}\) h\(^{-1}\) and showed a strong dependence on the level of K\(^+\) provision during growth (Fig. 24A–C). Thus, the intensity of transmembrane K\(^+\) transport appears to depend on plant K\(^+\) status in the low-affinity range, a property previously thought to be exclusive to the high-affinity range (Glass and Dunlop 1978; Kochian and Lucas 1982; Gierth et al. 2005). However, this variability in V\(_{\text{max}}\) probably does not reflect changes in transporter-gene expression (as is known to occur in the high-affinity range; Gierth et al. 2005) but rather may be the result of the well-documented effects of plant K\(^+\) status on the membrane electrical polarization that drives the thermodynamically passive, channel-mediated flux (Szczerba et al. 2009). In Arabidopsis athak5 atakt1 double-knock-out mutants,
which we used in conjunction with inhibitors to bring about a more complete cessation of transmembrane K\(^+\) transport, we still observed a linearly rising influx after inhibition. Subtraction of this inhibitor-insensitive component from the control flux revealed a transmembrane flux saturating at \(\sim 13 \, \mu\text{mol g}^{-1} \text{h}^{-1}\) (Fig. 24D) indicating a moderate capacity of the genetically as-yet-undefined “back-up system” (BUS) for K\(^+\) uptake (Pyo et al. 2010; Caballero et al. 2012; Coskun et al. 2013c). The relatively high transmembrane flux in the double mutants can, in part, be explained thermodynamically by the highly negative plasma-membrane potentials in their root cells (below \(-250\) mV; Coskun et al. 2013c; Subchapter 4.1). Indeed, this was the explanation proposed for very high (25 \(\mu\text{mol g}^{-1} \text{h}^{-1}\), at 5 mM [K\(^+\)]\(_{\text{ext}}\)), malleable, and short-lived K\(^+\) fluxes observed in barley plants, upon withdrawal of NH\(_4\)\(^+\) supply (Coskun et al. 2013c; Subchapter 4.1). Interestingly, we observed broadly similar fluxes (with and without inhibitors) in Col-0 wildtype seedlings compared to the double mutant (not shown), suggesting that the BUS compensates (perhaps completely) for the lack of AtHAK5 and AtAKT1 in the [K\(^+\)]\(_{\text{ext}}\) range tested. Although beyond the scope of this study, a thorough characterization of the BUS, involving its kinetics, selectivity, regulation, and genetic candidate(s), is warranted (see Pyo et al. 2010; Caballero et al. 2012; Coskun et al. 2013c for early indications).

Unifying influx and efflux data, we propose that, as ion concentrations rise, the increased apoplastic tracer retention and cycling can account for a persistent and increasing portion of the measured tracer fluxes, giving rise to linearly rising influx isotherms, which can indeed continue to rise \textit{ad absurdum} (Fig. S1), to levels that are neither energetically probably nor compatible with the limits of cellular membrane integrity (Britto et al. 2010, and references therein). Our data suggest that the extremely high LATS fluxes often reported with short labeling-time protocols (Essah et al. 2003; Malagoli et al. 2008; Wang et al. 2009) are largely attributable to apoplastic “noise”, which is then multiplied many-fold when extrapolating influx values to a per-hour flux (\textit{e.g.} 60-fold for 1-min uptake measurements, or 30-fold for the commonly recommended 2-min protocols; Essah et al. 2003; Fig. 22C). It therefore became apparent that the recommendations for very short labeling and desorption times under low-affinity conditions, due to high rates of efflux from root cells (see above), are unfounded.

A new problem this proposal raises, however, concerns the structural and functional nature of this apoplastic fraction. It does not represent a component of the “Donnan space” of cell walls, for example, as it does not display ion-exchange characteristics, \textit{i.e.} K\(^+\) ions are not displaceable
by strong divalent cations, such as \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \) (not shown, but see Fig. 24D), and it shows desorption kinetics slower than what are usually attributed to the Donnan phase of the apoplast (Kronzucker et al. 1995), but similar to other efflux phases verified as arising from the cytosol, at lower substrate concentrations, in other experimental works (Kronzucker et al. 1995; see also Fig. 23A and Table 9). Clearly, the problematic structure and function of this apoplastic phase in roots requires further investigation.

The contribution of non-transmembrane transport also pertains to long-distance transport to the shoot. Fig. S3 shows an experiment in which we stepped up the external supply of \( \text{K}^+ \) to roots of intact plants, and observed a significant rise in the contribution of \(^{42}\text{K}^+\) accumulation in the shoot relative to the total accumulation in the plant. We suggest, based on inhibitory treatments (Fig. 24B), that this rising shoot flux also has a significant apoplastic component, an idea which agrees with a substantial body of literature showing there to be an “apoplastic bypass flow” from root to shoot in the low-affinity range, especially well-demonstrated in the case of \( \text{Na}^+ \) translocation in rice, but also observed with potassium and in other plant species (Yeo and Flowers 1984; Yeo et al. 1987). Such long-distance fluxes can contribute additionally to influx isotherms in cases where whole plants are examined, and can be misconstrued as representing fractions of influx across cellular membranes. Generally, however, these are minor contributions to overall influx, as shoot translocation represents a small fraction of net accumulation in short-term tracer studies, and is typically small compared to total unidirectional influx and efflux in roots (as manifest in the high efflux:influx ratios at high substrate concentrations; see above). The largest contributor to misattributed influxes in the LATS range, therefore, is an apoplastic “bypass” within the root system itself, which has, hitherto, gone unnoticed. In further support of this idea, some of the highest LATS-range ion fluxes on record (\( \text{Na}^+ \) fluxes in Arabidopsis), were also obtained in excised roots, lacking a shoot system altogether (Essah et al. 2003). We have previously noted that it is unlikely that these fluxes proceed across the plasma membrane, on energetic grounds (Britto and Kronzucker 2009).

In summary, we propose to revise the model for \( \text{K}^+ \) transport in roots of higher plants (Fig. 26). Due to the negligible contribution of apoplastic fluxes at low \([\text{K}^+]_{\text{ext}}\) (e.g. <1 mM), it is clear that tracer-influx measurements accurately reflect the activities of high-affinity transporters (e.g. \( \text{AtHAK5} \) and \( \text{HvHAK1} \)) in this range. Under intermediate LATS conditions (i.e. 1–10 mM \([\text{K}^+]_{\text{ext}}\)), the activities of low-affinity channels (e.g. \( \text{AtAKT1}, \text{HvAKT1}, \) and \( \text{BUS} \)) mediating...
transmembrane influx can also be gauged by tracer analysis, but only if distortions brought about by apoplastic tracer retention are taken into account. Some contribution to transmembrane influx in this range might also occur via high-affinity transporters (e.g. AtHAK5 or HvHAK1), which can display dual-affinity kinetics (Fu and Luan 1998); however, their contribution is likely to be negligible, given that the prevailing thermodynamic conditions favour passive influx across the membrane (Table S2), and that the expression of these transporters is down-regulated even at moderate \([\text{K}^+]_{\text{ext}}\) (Gierth and Mäser 2007). Under high LATS conditions (i.e. 10–100 mM \([\text{K}^+]_{\text{ext}}\)), however, the standing model needs major revision, with transmembrane systems becoming saturated, and linear apoplastic fluxes beginning to dominate (Fig. 26). It is critical to recognize the contribution of apoplastic fluxes in this range, in order to accurately discern transmembrane events and attribute them to the expression of specific genes and the activities of specific gene products.

Lastly, but perhaps most significantly, we believe that our model may also pertain to the linear nature of low-affinity fluxes for other ions, such as \(\text{NH}_4^+\), \(\text{NO}_3^-\), and \(\text{Cl}^-\), and, most importantly, \(\text{Na}^+\), in the context of salinity stress. \(\text{Na}^+\) influx into plant roots has been shown to display a strong linear response to external \([\text{Na}^+]\) throughout the saline range in several studies (Kronzucker and Britto 2011; and references therein; see also Fig. S4), but, if the arguments presented here for \(\text{K}^+\) (and elsewhere; Britto and Kronzucker 2009) apply to \(\text{Na}^+\) as well, such fluxes may also be found to be apoplastic (Britto and Kronzucker 2015). Indeed, we believe that the lack of progress in identifying transporters for \(\text{Na}^+\) in the saline range (Kronzucker and Britto 2011) is, in part, related to a persistent misattribution of \(\text{Na}^+\) fluxes to transmembrane events. The demonstration of a substantial apoplastic \(\text{Na}^+\)-transport artefact could lead the way to the discovery of genuine membrane transport mechanisms in this critically important case.
Figure 26. The revised model of K⁺ acquisition in roots of higher plants. The apparent flux measured with radiotracer (solid black line) is composed of a saturable transmembrane flux (red dashed line) and a linear extracellular (apoplastic) flux (black dotted line). The magnitude of the transmembrane flux is regulated by plant K⁺ status. The apoplastic contribution, by contrast, is strongly dependent on measurement protocol (i.e. labeling and desorption time).
5.1 Silver Ions Disrupt K⁺ Homeostasis and Cellular Integrity in Barley (*Hordeum vulgare* L.) Roots

Preface

A modified version of this chapter was published as: Coskun D, Britto DT, Jean Y-K, Schulze L, Becker A, Kronzucker HJ (2012) Silver ions disrupt K⁺ homeostasis and cellular integrity in barley (*Hordeum vulgare* L.) roots. *Journal of Experimental Botany* 63, 151-162. Supplementary material cited in this chapter can be found online: http://jxb.oxfordjournals.org/content/63/1/151/suppl/DC1.

HJ Kronzucker designed the study, with input from DT Britto, D Coskun, and A Becker. D Coskun, Y-K Jean, L Schulze, and A Becker performed the experiments and analyzed the data, with input from DT Britto and HJ Kronzucker. DT Britto and D Coskun wrote the manuscript, with input and editing from HJ Kronzucker.

5.1.1 Abstract

The heavy metals silver, gold, and mercury can strongly inhibit aquaporin-mediated water flow across plant cell membranes, but critical examinations of their side effects are rare. Here, the short-lived radiotracer 

\[ {^{42}}K \]

is used to demonstrate that these metals, especially silver, profoundly change potassium homeostasis in roots of intact barley (*Hordeum vulgare* L.) plants, by altering unidirectional K⁺ fluxes. Doses as low as 5 µM AgNO₃ rapidly reduced K⁺ influx to 5% that of controls, and brought about pronounced and immediate increases in K⁺ efflux, while higher doses of Au³⁺ and Hg²⁺ were required to produce similar responses. Reduced influx and enhanced efflux of K⁺ resulted in a net loss of >40% of root tissue K⁺ during a 15-min application of 500 µM AgNO₃, comprising the entire cytosolic potassium pool and about a third of the vacuolar pool. Silver also brought about major losses of UV-absorbing compounds, total electrolytes, and NH₄⁺. Co-application, with silver, of the channel blockers Cs⁺, TEA⁺, or Ca²⁺,
did not affect the enhanced efflux, ruling out the involvement of outwardly-rectifying ion channels. Taken together with an examination of propidium-iodide staining under confocal microscopy, the results indicate that heavy-metal aquaporin inhibitors affect K⁺ homeostasis by directly inhibiting K⁺ influx at lower concentrations, and indirectly inhibiting K⁺ influx and enhancing K⁺ efflux, via membrane destruction, at higher concentrations. Ni²⁺, Cd²⁺, and Pb²⁺, three heavy metals not generally known to affect aquaporins, did not enhance K⁺ efflux or cause propidium-iodide incorporation. The study reveals strong and previously unknown effects of major aquaporin inhibitors and recommends caution in their application.

5.1.2 Introduction

Aquaporins (AQPs) comprise a diverse, recently discovered group of membrane-bound proteins that facilitate the movement of water, and of small, usually uncharged molecules such as urea, glycerol, carbon dioxide, and ammonia, across the membranes of living cells (Maurel et al. 2008; Hove and Bhave 2011). In plants and other organisms, AQP function has been studied extensively by use of mercury (Hg²⁺)-containing compounds, which inhibit water flux at both the tissue and cellular level (Preston et al. 1992; Maurel et al. 1993). More recently, it has been shown that silver (Ag⁺) and gold (Au³⁺) ions can block the flow of water through AQPs even more effectively than mercury, albeit by a different mechanism (Niemietz and Tyerman 2002; see also Table S1). Silver is also used widely as an inhibitor in the study of ethylene biosynthesis (Beyer 1976; see also Table S1). As with the use of any inhibitory treatment, it is crucial to determine the specificity of the inhibition. The toxic nature of mercury is legendary (Patra and Sharma 2000; Eisler 2006), and Santoni et al. (2000) cautioned that its disruption of cellular metabolism and solute homeostasis could cause confounding side effects such as the downregulation of AQP activity, or the collapse of water potential gradients across cell membranes. These authors concluded that mercury could, nevertheless, be reasonably applied in water transport studies, provided that low concentrations (<100 µM) are used, and that control experiments are conducted to indicate that perturbations to cellular functions, such as ion transport, are minimal (see also Meharg and Jardine 2003). Similarly, Zhang and Tyerman (1999) suggested that high concentrations of mercury (300 µM) could have non-specific,
detrimental effects on cell membranes, and that lower concentrations should therefore be employed in AQP studies.

In practice, however, such control experiments can be difficult, and are rarely conducted. In addition, as can be seen in Table S1, which shows the result of a literature survey on the use of mercury, silver, and gold as AQP inhibitors (and, in the case of silver, also as an inhibitor of ethylene biosynthesis), applied concentrations of several hundred micromolar, or even several millimolar, are frequently used, well in excess of the (somewhat arbitrary, as we shall see) limit suggested by Santoni et al. (2000).

In the present authors’ own laboratory, AQP function has recently been investigated due to a long-standing interest in the mechanisms of N uptake in plant cells in the context of the hypothesis that low-affinity \( \text{NH}_3/\text{NH}_4^+ \) transport into root cells is mediated by AQPs. While there is growing evidence for such mediation in heterologous expression systems (Jahn et al. 2004; Holm et al. 2005; Loqué et al. 2005; Hove and Bhave 2011), a convincing \textit{in planta} demonstration is still lacking. The initial hypothesis was supported by the strong reduction, by mercury and silver, of \( ^{13}\text{N} \)-labelled \( \text{NH}_3/\text{NH}_4^+ \) influx into barley roots, but, surprisingly, was then contradicted by a potent silver-induced stimulation of \( ^{13}\text{N} \) efflux.

These somewhat paradoxical findings led to a new hypothesis, that the effects of silver, and possibly other heavy-metal inhibitors, on tracer fluxes extend beyond a simple inhibition of AQP activity. In the present study, this hypothesis was tested by examination of the effects of \( \text{Hg}^{2+}, \text{Ag}^+, \text{and Au}^{3+} \) on unidirectional fluxes (root influx and efflux) of \( \text{K}^+ \), an ion not transported to a significant extent by AQPs (Agre et al. 1997). As will be shown, the results led to a new research question: is membrane integrity disrupted in roots treated with AQP inhibitors? This possibility, similar to one previously put forward to explain sodium-stimulated \( \text{K}^+ \) efflux (Britto et al. 2010), was investigated here using a wide variety of methods, including measurements of tracer fluxes, release of UV-absorbing compounds and electrolytes, tissue ion content, and confocal microscopy.
5.1.3 Results

Figure 27 shows measurements of unidirectional $^{13}$N-radiolabelled NH$_3$/NH$_4^+$ influx into intact barley seedlings, with and without a 10-min treatment of 500 µM AgNO$_3$. Silver application reduced $^{13}$N influx to less than half that of control, while, in the opposite direction of transport, it brought about an immediate and substantial stimulation of $^{13}$N efflux from pre-labelled roots (Fig. 27, inset). Similar, if even more pronounced, effects of AgNO$_3$ were observed when tracing unidirectional potassium influx using $^{42}$K$^+$, with concentrations as low as 5 µM reducing influx by 95% (Fig. 28). A small subset of experiments involving 5 µM silver sulphadiazine also showed a dramatic suppression of K$^+$ influx (Fig. S1). In addition, treatment with Au$^{3+}$ and Hg$^{2+}$, two other heavy metals known to inhibit AQPs (Niemietz and Tyerman 2002), essentially abolished K$^+$ influx at 500 µM, while lower concentrations also showed significant reductions in influx, particularly with mercury (Fig. 28). In contrast, Cd$^{2+}$ and Pb$^{2+}$, heavy metals described as non-AQP inhibitors (Niemietz and Tyerman 2002), brought about only a slight inhibition of K$^+$ influx, far less than Ag$^+$, Au$^{3+}$, and Hg$^{2+}$ (Fig. 28). However, Ni$^{2+}$, though also not identified as an AQP inhibitor (Niemietz and Tyerman 2002), did significantly reduce K$^+$ influx to a similar extent as Ag$^+$, Au$^{3+}$, and Hg$^{2+}$ (Fig. 28). As with $^{13}$N, stimulation of $^{42}$K$^+$ efflux was also observed. Application of 500 µM Ag$^+$ was the most powerful stimulatory treatment, followed by 50 µM Ag$^+$, while 500 µM Au$^{3+}$ and Hg$^{2+}$ stimulated K$^+$ release to an extent similar to 5 µM Ag$^+$ (Fig. 29A). $^{42}$K$^+$ efflux also showed an immediate and substantial acceleration due to 5 µM silver sulphadiazine, an identical response to that of equimolar AgNO$_3$ (Fig. S1, inset). The dose dependence of AgNO$_3$ revealed a ~2-fold increase in tracer released with a 10-fold increase in applied silver concentration (Fig. 29A, internal legend). Interestingly, K$^+$ efflux stimulation by 160 mM Na$^+$, a well-studied phenomenon (Nassery 1979; Shabala et al. 2006; Britto et al. 2010), was modest compared with these treatments, with tracer release falling below that seen with 5 µM silver. Applications of Au$^{3+}$ and Hg$^{2+}$, at lower concentrations (5 µM or 50 µM), did not result in $^{42}$K$^+$ efflux stimulation, nor did the application of the organic AQP modulators acetazolamide, zonisamide, or forskolin (all at 10 µM) (Fig. 29B). Applications of 500 µM Pb$^{2+}$, Cd$^{2+}$, and Ni$^{2+}$ produced little change to the efflux of K$^+$ compared with control (Fig. 29C).
Figure 27. Response of $\text{NH}_3/\text{NH}_4^+$ influx into roots of intact barley (*Hordeum vulgare* L.) seedlings to 10-min incubation in 500 µM $\text{Ag}^+$. Inset: response of $^{13}\text{NH}_3/^{13}\text{NH}_4^+$ efflux from roots of intact barley seedlings to sudden provision (at elution time $t = 15$ min, see arrow) of 500 µM $\text{Ag}^+$. Asterisk represents a significantly different mean from control (t-test, $P < 0.05$). Each treatment represents the mean of three replicates. Error bars indicate ± SEM.
**Figure 28.** Response of K⁺ influx into roots of intact barley (*Hordeum vulgare* L.) seedlings to 10-min incubation in Ag⁺, Hg²⁺, and Au³⁺ (at 5, 50, and 500 µM), and Pb²⁺, Cd²⁺, and Ni²⁺ (at 500 µM). Asterisks represent significantly different means from control (one-way ANOVA with Dunnett’s post-test). Each treatment represents the mean of 4–7 replicates. Error bars indicate ± SEM.
Figure 29. Response of $^{42}\text{K}^+$ efflux from roots of intact barley (*Hordeum vulgare* L.) seedlings to sudden provision (see arrow) of (A) Ag$^+$, Hg$^{2+}$, and Au$^{3+}$ (at 5, 50, and 500 µM), and 160 mM Na$^+$; (B) 10 µM acetazolamide, zonisamide, and forskolin; (C) 500 µM Pb$^{2+}$, Cd$^{2+}$, and Ni$^{2+}$. In the internal legend, the numbers in parentheses indicate the amount of K$^+$ released during a specific treatment ($t = 16.5$–$29.5$ min), in µmol g$^{-1}$ (root FW). Each plot represents the mean of 3–8 replicates. Error bars indicate ± SEM.
To address the hypothesis that the observed stimulation of efflux was mediated by K⁺ channels or non-selective cation channels (NSCCs), the K⁺ channel blocking agents Cs⁺ and TEA⁺ (both at 10 mM), and the NSCC blocker Ca²⁺ (at 5 mM) were co-applied with 500 µM Ag⁺ (Fig. 30). None of these channel blockers significantly attenuated the stimulation of efflux observed with 500 µM Ag⁺ (P < 0.05). Moreover, no reduction by 10 mM Cs⁺ of the stimulated efflux induced by 5 µM Ag⁺ was observed, whereas when 5 µM Au³⁺ and Hg²⁺ were co-applied with 10 mM Cs⁺, a depression of efflux below control values was observed (not shown). We should note that Cs⁺ was applied as the chloride salt here to avoid precipitation (similarly, sulphate salts were used in the case of Ag⁺ application).
**Figure 30.** Response of $^{42}$K$^+$ efflux from roots of intact barley (*Hordeum vulgare* L.) seedlings to sudden provision (see arrow) of 500 µM Ag$^+$ in combination with the channel inhibitors Cs$^+$ (10 mM, as Cs$_2$SO$_4$), TEA$^+$ (10 mM, as TEA-NO$_3$), and Ca$^{2+}$ (5 mM, as CaSO$_4$). Each plot represents the mean of 3–8 replicates. Error bars indicate ± SEM.
To test the alternative hypothesis that the effects of silver, gold, and mercury on ion fluxes are related to membrane integrity, two independent assays of membrane leakiness were conducted. Table 10 shows the relative leakage ratio of control and silver-treated roots, as determined by measuring either electrical conductivity or UV absorbance of the bathing medium, before and after autoclaving the roots. Both assays showed that a 15-min incubation of intact roots in 500 µM Ag⁺ significantly increased the relative leakiness of cellular membranes, suggesting that this treatment caused membrane damage. It should be noted that the measurements were taken after silver treatment was terminated; that is, after substantial loss of cellular materials had already occurred. Therefore, these standard protocols underestimated the difference between treatment and control (Table 10).

Changes in ionic content of roots following silver application were also measured, by photometric analyses of tissues (Table 10). Root-K⁺ content decreased by 43% and NH₄⁺ content decreased by 69% after a 15-min application of 500 µM Ag⁺, further suggesting that extensive membrane damage had taken place. When plants exposed to 500 µM Ag⁺ for 15 min were returned to normal growth solution for 1 week, root growth cessation and shoot growth suppression was observed over the following 6 d (not shown), showing that silver brought about irreversible damage.

For comparison with total K⁺ loss from tissue, the tracer released following 500 µM Ag⁺ treatment in ⁴²K⁺ efflux experiments was summed and expressed in terms of micromoles. Analysis showed that treated roots lost a minimum of 4.38 µmol K⁺ g⁻¹ over a 15-min period (Fig. 31; note that these curves were truncated slightly prematurely; a small set of experiments was conducted wherein elution was extended for an additional 30 min, resulting in an additional 25% of tracer loss).

Figure 32 directly illustrates the membrane damage brought about by 5 µM and 500 µM Ag⁺ (Fig. 32B and C, respectively), 500 µM Hg²⁺ (Fig. 32D), and 500 µM Au³⁺ (Fig. 32E), by means of propidium-iodide staining visualized with confocal microscopy. Compared with control (Fig. 32A), where only cell walls were stained, all regions of the root tip showed nuclear staining (and, by inference, membrane disruption) when intact seedlings were incubated for 15 min with the AQP inhibitors. In contrast, roots treated for 15 min with the heavy metals Cd²⁺, Ni²⁺, and Pb²⁺ (all at 500 µM) (Fig. 32F–H, respectively) lacked nuclear staining, indicating lack of membrane
damage. Root tips treated with 160 mM NaCl for the same duration also showed no nuclear staining (Fig. 32I).

Pressure chamber measurements were conducted to test the effect of Ag⁺ and Au³⁺ on the water potential of intact seedling roots. Fig. S2 shows that a 15-min treatment with 500 µM Ag⁺ or Au³⁺ significantly impeded water flow in roots of experimental plants.
Table 10. Effects of Ag⁺ on the relative leakage ratio (RLR), and K⁺ and NH₄⁺ contents, of roots from 7-day-old barley (*Hordeum vulgare* L.) seedlings. Errors indicate ± SEM of 6–12 replicates. Asterisks indicate significant differences from control (t-test, P <0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC (µS)</th>
<th>A₂₈₀ (AU)</th>
<th>Root K⁺ content (µmol g⁻¹ FW)</th>
<th>Root NH₄⁺ content (µmol g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>75.74 ± 4.48</td>
<td>221.65 ± 20.72</td>
<td>0.10 ± 0.02</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RLR = 0.36 ± 0.02</td>
<td>RLR = 0.17 ± 0.04</td>
</tr>
<tr>
<td>500 µM AgNO₃</td>
<td>21.20 ± 0.56</td>
<td>34.4 ± 1.08</td>
<td>0.12 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RLR = 0.62 ± 0.02*</td>
<td>RLR = 0.61 ± 0.03*</td>
</tr>
</tbody>
</table>
Figure 31. Illustration of the integration technique employed to quantify total K\(^+\) released during treatment with 500 \(\mu\)M Ag\(^+\). As indicated in the equation, K\(^+\) released is determined by the summation of counts released per gram during Ag\(^+\) treatment (shaded area), divided by the corrected specific activity of the cytosol, where SA\(_{o}\) represents the specific activity of the loading solution, \(k\) the rate constant representing the slope of the semi-logarithmic regression line of the slowest exchanging (cytosolic) phase, \(t\) the loading time (60 min), and \(t'\) the time between the start of the elution series and the beginning of the treatment (15.5 min). Based on eight replicates, 4.38 ± 0.22 \(\mu\)mol g\(^{-1}\) was released from seedlings treated with 500 \(\mu\)M Ag\(^+\) for 15 min. Note that this graph is prematurely truncated; however, a few experiments involving a longer term (45-min) treatment and elution were conducted, and showed that ~25% additional K\(^+\) was available for release.
Figure 32. Confocal micrographs showing propidium iodide staining of the cell wall and nuclei of damaged cells from lateral root tips of intact barley (*Hordeum vulgare* L.) seedlings treated for 15 min in (A) control, (B) 5 µM Ag⁺, (C) 500 µM Ag⁺, (D) 500 µM Hg²⁺, (E) 500 µM Au³⁺, (F) 500 µM Cd²⁺, (G) 500 µM Ni²⁺, (H) 500 µM Pb²⁺, and (I) 160 mM Na⁺. Scale bars represent 20 µm.
5.1.4 Discussion

In 2002, Niemietz and Tyerman demonstrated that silver, gold, and mercury ions reduced osmotically induced shrinking of human erythrocytes and of vesicles composed of root plasma membrane of beet or peribacteroid membrane from soybean. Silver was particularly effective in this regard, followed by gold and then mercury. Because the osmotic shrinking response of cells and vesicles is directly linked to water permeability, the authors concluded that silver is a potent AQP inhibitor, much more so than mercury, which has been, and continues to be, widely used as a blocker in AQP studies (see Table S1). This discovery may help explain why silver is effective as an antimicrobial agent (Kim et al. 2008) and in the preservation of cut flowers (Beyer 1976; Lue et al. 2010).

In the present study, it was shown that when Ag\(^+\), Au\(^{3+}\), and Hg\(^{2+}\) are applied to roots of intact barley plants, pronounced effects on transmembrane ion fluxes and membrane integrity occur in addition to the reduction in water flux. Initially, it was observed that Ag\(^+\) stimulated the efflux of \(^{13}\)N-labelled NH\(_3\)/NH\(_4\)\(^+\) from roots (Fig. 27, inset), but follow-up experiments indicated that the effect is more general, with Ag\(^+\) also increasing efflux of labelled potassium, even at concentrations as low as 5 \(\mu\)M Ag\(^+\) (Fig. 29A). In addition, 500 \(\mu\)M Au\(^{3+}\) and Hg\(^{2+}\) also stimulated K\(^+\) release, but the same metals applied at lower concentrations (5 \(\mu\)M and 50 \(\mu\)M) showed no effect, in contrast to Ag\(^+\) (Fig. 29A). Because the K\(^+\) channel blockers TEA\(^+\) and Cs\(^+\), and the NSCC blocker Ca\(^{2+}\), failed to reduce the stimulation of K\(^+\) efflux by 500 \(\mu\)M Ag\(^+\) (Fig. 30), and similar results were seen with 5 \(\mu\)M Ag\(^+\) plus Cs\(^+\) (not shown), the possibility was ruled out that this effect is due to increased activity of ion channels in root cell membranes, an explanation put forward for the NaCl-stimulated efflux of potassium in the work of Shabala et al. (2006) (see also Iwabuchi et al. 2008; cf. Britto et al. 2010). On the other hand, when 10 mM Cs\(^+\) was co-applied with 5 \(\mu\)M Au\(^{3+}\) or Hg\(^{2+}\), an inhibition of efflux was observed (not shown), similar to what was seen in a previous study, in which 10 mM Cs\(^+\) suppressed steady-state K\(^+\) efflux in the same cultivar of barley and at the same external [K\(^+\)] (Coskun et al. 2010). These observations suggest that membranes are intact, and outward rectifying channels are functional under these conditions, unlike at high (500 \(\mu\)M) concentrations of Au\(^{3+}\) and Hg\(^{2+}\) and all tested concentrations of Ag\(^+\).
In contrast to the present finding, MacRobbie (2006) suggested that one component of stimulated K\textsuperscript{+} release from vacuoles of guard cells, as measured by \textsuperscript{86}Rb\textsuperscript{+} efflux, is stimulated by the AQP inhibitors mercury and silver, and mediated by ion channels. However, membrane intactness was not determined in that study, and the present results may offer an alternative explanation for the stimulation. It should also be noted that thresholds and degrees of sensitivity to inhibitors do vary among experimental systems. This is apparent from Table S1, which shows that Ag\textsuperscript{+}, Au\textsuperscript{3+}, and Hg\textsuperscript{2+} do not inhibit water flow in all cases. In addition, some studies using the cell pressure probe have indicated that cellular turgor is maintained in the presence of these metals, which would not be possible were significant membrane damage to have occurred (Zhang and Tyerman 1999; Tazawa et al. 2001; Hukin et al. 2002; Bramley et al. 2009).

Based on additional experimentation showing silver-induced losses of electrolytes and UV-absorbing compounds from barley roots (Table 10), as well as confocal microscopy analysis showing membrane damage resulting from 500 µM Au\textsuperscript{3+} and Hg\textsuperscript{2+}, and all tested Ag\textsuperscript{+} concentrations (Fig. 32B–E), the present study strongly suggests that the stimulation of ion efflux due to these treatments is a direct result of membrane destruction. The suppression of ion influx (Figs. 27 and 28) can also be partly explained in this way; cells with a compromised membrane barrier and high efflux cannot be expected to retain tracer to the same extent as cells with intact membranes. It is interesting to note that the potency of the 500 µM Ag\textsuperscript{+} treatment was so great that co-application of 5 mM Ca\textsuperscript{2+} failed to protect membranes, as is sometimes observed with more benign treatments such as NaCl (Cramer et al. 1985; Britto et al. 2010). In contrast, the inhibition of influx observed under low (5 µM and 50 µM) Au\textsuperscript{3+} and Hg\textsuperscript{2+} (Fig. 28) cannot be explained by accelerated efflux or membrane damage, since no efflux stimulation was seen (Fig. 29A). These observations point toward a direct inhibition of K\textsuperscript{+} influx pathways brought about by these lower concentrations of Au\textsuperscript{3+} and Hg\textsuperscript{2+}, possibly via effects on cellular energetics (e.g. Martínez-Ballesta et al. 2003). In contrast to the heavy-metal AQP inhibitors, the organic AQP inhibitors acetazolamide and zonisamide, and the stimulator forskolin, showed no effect on K\textsuperscript{+} efflux in barley roots (Fig. 29B), suggesting that the stimulatory effects observed are specific to heavy-metal inhibitors. Further experimentation will need to be conducted to explore these novel findings. Moreover, the heavy metals used here that did not modify water relations in Niemietz and Tyerman (2002), namely nickel, cadmium, and lead, also had minimal effects on ion efflux in barley roots (Fig. 29C), and did not appear to cause membrane damage as determined by
confocal microscopy (Fig. 32F–H). Curiously, however, K$^+$ influx was moderately inhibited by Cd$^{2+}$ and Pb$^{2+}$ and strongly inhibited by Ni$^{2+}$ (Fig. 28); reasons for this are presently unclear, but may also be related to cellular energetics. The data demonstrate the clear distinction between heavy metals that block AQP$\text{s}$ and those that do not, strongly corroborating the findings of Niemietz and Tyerman (2002). The data also provide an alternative interpretation of the recent (and unexplained) observation that silver nitrate (≤10 µM) stimulates the release of indole-3-acetic acid from roots of Arabidopsis thaliana, independently of its effect on ethylene signalling (Strader et al. 2009).

As in the study of Niemietz and Tyerman (2002), the effect of Ag$^+$ was the most potent, followed distantly by Au$^{3+}$ and Hg$^+$ (Fig. 29A). Indeed, when the dose dependence of Ag$^+$ was investigated, it was found that a 5-µM application had an effect approximately equal to that seen at 500 µM Hg$^{2+}$ and Au$^{3+}$. This Ag$^+$ concentration was as effective whether applied as AgNO$_3$ or silver sulphadiazine, a result in contrast to the finding of Niemietz and Tyerman (2002), who showed a greater effect of silver sulphadiazine on water flow (Fig. S1). Interestingly, the stimulation of K$^+$ efflux by high provision of NaCl, an effect that has been extensively investigated by numerous groups (e.g. Nassery 1979; Shabala et al. 2006; Britto et al. 2010), was less pronounced, and less protracted, than that of any of the heavy-metal AQP inhibitors examined here. It had previously been proposed (Britto et al. 2010) that NaCl-induced K$^+$ efflux was also a result of membrane disintegrity, but, in the present study, confocal micrographs of roots stained with propidium iodide did not reveal such damage (Fig. 32I). This may indicate that the destructive effect of NaCl on membranes is below the threshold for entry of this large fluorescent molecule into the cell, a suggestion consistent with the relatively small stimulation of K$^+$ efflux by NaCl, compared with the heavy-metal AQP inhibitors, despite an orders-of-magnitude greater applied concentration. Another possibility is that membrane disintegrity when NaCl is applied may be rapidly reversible through re-annealing of the lipid bilayer, as suggested by the observation of rapid recovery from an initial wilting response to NaCl (not shown). This could conceivably occur through the regulated response of AQPs to osmotic shock, a response not possible in the presence of AQP inhibitors.

It was of further interest to quantify the ionic release from roots treated with silver. Analysis showed that 500 µM Ag$^+$ application brought about the release of ~5 µmol of K$^+$ per gram of root (Fig. 29A, internal legend; and Fig. 31). If all the $^{42}$K$^+$ released were to have originated in
the cytosol of root cells, and if all the cytosolic $K^+$ were released after silver application, this value would correspond to an approximate cytosolic [K+] of 100 mM, considering that cytosolic volume is ~5% of tissue volume (Lee and Clarkson 1986), and 1 g of root has a volume of ~1 ml. This amount is remarkably close to cytosolic [K+] values measured in a large number of studies (for a review, see Britto and Kronzucker 2008) using techniques as diverse as non-aqueous fractionation (Speer and Kaiser 1991), longitudinal ion profiling (Jeschke and Stelter 1976), X-ray microanalysis (Flowers and Hajibagheri 2001), fluorescing dyes (Halperin and Lynch 2003), ion-selective intracellular microelectrode analysis (Walker et al. 1996a), and compartmental analysis by tracer efflux (Pitman and Saddler 1967; Kronzucker et al. 2003). Considering that labelling times in the present study were restricted to 60 min, and that vacuolar half-times of exchange for $K^+$ are in the order of 12–20 h (Memon et al. 1985), contributions from the vacuole to the tracer-release spikes should be negligible (Britto and Kronzucker 2001). Nevertheless, substantial loss from the vacuole is also sustained during a 15-min application of 500 µM silver, as demonstrated by tissue analysis using flame photometry, which showed that ~40% of total root $K^+$ is lost (Table 10). Because of these considerations of labelling kinetics and tissue accumulation, therefore, the loss of cytosolic $K^+$ appears to be essentially complete, while the majority of vacuolar $K^+$ is retained. Given the importance of $K^+$ as a major plant nutrient, such a massive loss may be sufficient to explain the cessation of growth in roots treated for 15 min (see above), and the eventual death of the plant when treated for 1 week (not shown). However, the concomitant loss of other intracellular components and the major membrane damage incurred by silver application are additional effects that probably contribute to the lethality of silver ions.

An important fundamental question remains. What causes the observed phenomena? Are the deleterious effects of heavy-metal AQP inhibitors on potassium homeostasis and root cell membranes of intact barley plants related purely to their blockage of water flux, or are there other, unrelated, physiological effects caused by these agents? Certainly, the observed inhibition of $K^+$ influx by low concentrations of Hg$^{2+}$ and Au$^{3+}$ and the concomitant lack of effect on efflux suggest the latter. Moreover, the lack of effect by the organic AQP modulators acetazolamide, zonisamide, and forskolin point towards a phenomenon more specific to heavy-metal blockers, rather than AQP modulators in general. It is interesting to note that the osmotic shrinking assays conducted by Niemietz and Tyerman (2002) did not appear to involve membrane damage in vesicles or erythrocytes, nor did Schreurs and Rosenberg (1982) find evidence that silver induces
cell damage or reduces cell viability in *Escherichia coli* cells, even though they observed stimulated efflux of accumulated phosphate, proline, glutamine, mannitol, and succinate. Similarly, Asharani et al. (2010) demonstrated that, while silver nanoparticles caused lysis and deterioration of human erythrocytes, silver ions alone showed no such effect. Moreover, it is well known that silver nitrate stimulates callus proliferation, shoot regeneration, and somatic embryogenesis in tissue cultures of many plant species, via its inhibition of ethylene action (Al-Khayri and Al-Bahrany 2001, and references therein). An open question is whether the elevated efflux and membrane destruction demonstrated here is linked to the scale and complex multicellular nature of the intact, transpiring barley plant, as compared with embryonic, single-celled or subcellular systems, such as callus cultures, *E. coli*, erythrocytes, or membrane vesicles, in which transpiration is limited or absent. Certainly, further experimentation is required to address these issues.

Finally, a note of caution is warranted regarding the use of heavy-metal AQP inhibitors, greatly extending the caveats of Santoni et al. (2000); see also Strader et al. (2009). The present investigation into the stimulated efflux observed upon application of heavy-metal AQP inhibitors stemmed from the examination of an entirely different problem: that of NH$_3$ penetration into cells via AQPs. Only after the focus was shifted to questions related to membrane transport and integrity, as well as K$^+$ homeostasis, did the severe side effects become apparent. These include membrane destruction brought about by high (500 µM) concentrations of Au$^{3+}$ and Hg$^{2+}$, and a wide range (5, 50, and 500 µM) of Ag$^+$ concentrations, rendering the cell incapable of retaining critical cellular constituents. Moreover, it was demonstrated that low (5 µM and 50 µM) concentrations of Au$^{3+}$ and Hg$^{2+}$ directly inhibit K$^+$ influx, without compromising cellular integrity. However, inhibition of K$^+$ uptake will have wide-ranging effects on numerous aspects of plant function (Britto and Kronzucker 2008), and, because K$^+$ is the most abundant cellular osmoticum, blockage of its uptake may in itself change water gradients in the longer term, confounding the use of heavy-metal AQP inhibitors in the study of plant water relations. The present study points to the need for a more discriminating use of inhibitors as a tool for physiological examination, particularly with heavy-metal AQP inhibitors *in planta.*
Chapter 6

NH₃/NH₄⁺ Transport and Toxicity: Mechanisms and Updates on the Futile-Cycling Hypothesis

6.1 Rapid Ammonia Gas Transport Accounts for Futile Transmembrane Cycling Under NH₃/NH₄⁺ Toxicity in Plant Roots

Preface

A modified version of this chapter was published as: Coskun D, Britto DT, Li M, Becker A, Kronzucker HJ (2013) Rapid ammonia gas transport accounts for futile transmembrane cycling under NH₃/NH₄⁺ toxicity in plant roots. *Plant Physiology* **163**, 1859-1867. Supplementary material cited in this chapter can be found online:

http://www.plantphysiol.org/content/163/4/1859.

HJ Kronzucker designed the study, with input from DT Britto and D Coskun. D Coskun, M Li, and A Becker performed the experiments and analyzed the data, with input from DT Britto and HJ Kronzucker. D Coskun wrote the manuscript, with input and editing from DT Britto and HJ Kronzucker.

6.1.1 Abstract

Futile transmembrane NH₃/NH₄⁺ cycling in plant root cells, characterized by extremely rapid fluxes and high efflux to influx ratios, has been successfully linked to NH₃/NH₄⁺ toxicity. Surprisingly, the fundamental question of which species of the conjugate pair (NH₃ or NH₄⁺) participates in such fluxes is unresolved. Using flux analyses with the short-lived radioisotope 

^{13}N and electrophysiological, respiratory, and histochemical measurements, we show that futile cycling in roots of barley (*Hordeum vulgare*) seedlings is predominately of the gaseous NH₃ species, rather than the NH₄⁺ ion. Influx of 

^{13}NH₃/^{13}NH₄⁺, which exceeded 200 μmol g⁻¹ h⁻¹, was not commensurate with membrane depolarization or increases in root respiration, suggesting electroneutral NH₃ transport. Influx followed Michaelis-Menten kinetics for NH₃ (but not NH₄⁺),
as a function of external concentration (K_M = 152 µM, V_max = 205 µmol g^{-1} h^{-1}). Efflux of \( ^{13}\text{NH}_3/^{13}\text{NH}_4^+ \) responded with a nearly identical K_M. Pharmacological characterization of influx and efflux suggests mediation by aquaporins. Our study fundamentally revises the futile-cycling model by demonstrating that \( \text{NH}_3 \) is the major permeating species across both plasmalemma and tonoplast of root cells under toxicity conditions.

6.1.2 Introduction

Ammonia/ammonium (\( \text{NH}_3/\text{NH}_4^+ \)) toxicity in higher plants has resulted in crop reduction and forest decline (Pearson and Stewart 1993; Vitousek et al. 1997; Britto and Kronzucker 2002), biodiversity loss (Stevens et al. 2004; Bobbink et al. 2010), and species extirpation (de Graaf et al. 1998; McClean et al. 2011). These major ecological and economic problems have been aggravated by an accelerated global nitrogen (N) cycle caused primarily by the industrialized production and use of N fertilizers (Gruber and Galloway 2008; Galloway et al. 2008). With increasing global population and demands on agricultural production, there is no sign of this trend easing: anthropogenic N fixation has reached 210 teragrams year^{-1}, an approximately 12% increase from 2005 and an approximately 1,300% rise from 150 years ago (Galloway et al. 2008; Fowler et al. 2013).

Although considerable knowledge of the causes and mechanisms of \( \text{NH}_3/\text{NH}_4^+ \) toxicity has accrued in recent years, our understanding of the key processes remains rudimentary (Gerendas et al. 1997; Britto and Kronzucker 2002). A major hypothesis is that of futile transmembrane \( \text{NH}_4^+ \) cycling, which proposes a pathological inability of root cells to restrict the primary entry of \( \text{NH}_4^+ \) at high external concentrations ([\( \text{NH}_4^+ \)]_{\text{ext}}); many downstream toxicological events are contingent upon this entry (Britto et al. 2001b). In this model, a rapid, thermodynamically passive influx of \( \text{NH}_4^+ \) is coupled to an active efflux of \( \text{NH}_4^+ \) that is nearly as rapid, constraining normal cellular function and energetics and resulting in plant growth decline and mortality. This phenomenon is thought to occur in \( \text{NH}_4^+ \)-sensitive species such as barley (\( \text{Hordeum vulgare} \)) and, to a lesser extent, in tolerant species such as rice (\( \text{Oryza sativa} \)), which can be susceptible at higher thresholds (Balkos et al. 2010; Chen et al. 2013).
Most soils are typically acidic, especially when $[\text{NH}_4^+]$ is high (i.e. in the millimolar range; Van Breemen et al. 1982; Bobbink et al. 1998; Britto and Kronzucker 2002), and given the pKa of 9.25 for the conjugate pair $\text{NH}_3/\text{NH}_4^+$, $[\text{NH}_3]$ is generally low (Izaurralde et al. 1990; Weise et al. 2013). Consequently, the fluxes of $\text{NH}_3$ have largely been considered negligible (Britto et al. 2001a; Britto and Kronzucker 2002; Loqué and von Wirén 2004), in contrast to $\text{NH}_4^+$ fluxes, which are well characterized physiologically (Lee and Ayling 1993; Wang et al. 1993a, b; Kronzucker et al. 1996) and at the molecular level (Rawat et al. 1999; von Wirén et al. 2000; Ludewig et al. 2007), at least at lower concentrations. However, the transport of $\text{NH}_3$ across membranes has received new attention in the light of evidence that some members of the aquaporin (AQP) family of transporters, a diverse and ubiquitous class of major intrinsic proteins (Maurel et al. 2008; Hove and Bhave 2011), can mediate $\text{NH}_3$ fluxes in single-cell systems (Jahn et al. 2004; Holm et al. 2005; Loqué et al. 2005; Saparov et al. 2007). However, a convincing demonstration that AQP s transport $\text{NH}_3$ in planta is currently lacking. Given the unusually high capacity of AQP-mediated fluxes relative to those of ion channels and other transporters (Kozono et al. 2002), it is possible that sizable $\text{NH}_3$ fluxes can be conducted through AQP s, even at very low external $\text{NH}_3$ concentration ($[\text{NH}_3]_{\text{ext}}$).

Here, we have critically re-examined the hypothesis that futile cycling is composed of cationic $\text{NH}_4^+$ fluxes across the plasmalemma, of which an active efflux mechanism accounts for energetic demands directly contributing to toxicity (Britto et al. 2001b). We present evidence for the following alternative scenario: 1) futile cycling consists mainly of the passive electroneutral flux of the conjugate base $\text{NH}_3$; 2) such fluxes rapidly span both major membrane systems in root cells (i.e. plasmalemma and tonoplast); 3) AQP s mediate such fluxes; and 4) a thermodynamic equilibrium of $\text{NH}_3$ is established throughout the cell, resulting in hyper-accumulation of $\text{NH}_4^+$ in the acidic vacuole. This evidence comes primarily from positron-emission tracing with the short-lived radioisotope $^{13}\text{N}$, used to characterize the component fluxes of futile cycling at the cellular level in the model species barley. We have coupled this with $^{42}\text{K}^+$ radiotracing, to provide comparison with a well understood cationic flux, as well as electrophysiological, respiratory, pharmacological, and histochemical analyses.
6.1.3 Results

To gauge the relative contributions of NH$_3$ and NH$_4^+$ transport, concentration-dependent root NH$_3$/NH$_4^+$ influxes and their associated plasma-membrane depolarization (change in membrane potential [$\Delta\Delta\Psi_m$]) were compared with those of the macronutrient ion potassium (K$^+$; Fig. 33A–C). In these experiments, plants were grown under non-toxic conditions, using a complete nutrient medium with K$^+$ and NH$_4^+$ both provided at 0.1 mM (pH 6.25). Direct influx measurements with $^{42}$K$^+$ and $^{13}$NH$_3$/$^{13}$NH$_4^+$, determined between 0.1 and 10 mM for each (with the other held constant at 0.1 mM), show influx of the two ions to have vastly different rates and isotherm shapes. For instance, steady-state NH$_3$/NH$_4^+$ influx (i.e. measured at the growth concentrations of 0.1 mM K$^+$ and NH$_4^+$) was 11.5-fold higher than that of K$^+$ (13.12 ± 0.85 versus 1.26 ± 0.06 µmol g$^{-1}$ h$^{-1}$, respectively). As each substrate’s concentration independently rose to 10 mM, its influx increased 4- to 5-fold, peaking with a NH$_3$/NH$_4^+$ influx 13-fold higher than that of K$^+$ (Fig. 33A). Interestingly, however, the rise in NH$_3$/NH$_4^+$ influx was not commensurate with membrane depolarization, indicating that most of the influx observed was not electrogenic and supporting the idea that NH$_3$, not NH$_4^+$, is the main transported N species. The significance of this result was underscored when compared with changes in $\Delta\Delta\Psi_m$ observed with K$^+$: increases in external K$^+$ concentration resulted in up to 4-fold greater depolarization than seen with comparable changes in NH$_3$/NH$_4^+$ (Fig. 33B), despite NH$_3$/NH$_4^+$ influx being more than 10-times higher than K$^+$ influx. Only at 10 mM were depolarizations of similar magnitude observed (approximately 60 mV). Figure 33C further illustrates this disproportion, by showing $\Delta\Delta\Psi_m$ as a function of influx for each substrate. The 9-fold steeper slope with K$^+$ relative to NH$_3$/NH$_4^+$ illustrates the much greater electrical response elicited by K$^+$ transport.

To further test the capacity of NH$_3$ transport in planta, we monitored NH$_3$/NH$_4^+$ influx as a function of each conjugate species’ external concentration independently, by adjusting solution pH and thus the [NH$_3$] to [NH$_4^+$] ratio (pKa = 9.25; Fig. 33D–F). Seedlings were grown under high (10 mM) external NH$_3$/NH$_4^+$ concentration ([NH$_3$/NH$_4^+$]$_{ext}$) in a full-nutrient medium (pH 6.25), then placed in growth solution with pH ranging between 4.25 and 9.25 for 10 min, prior to influx measurement with $^{13}$NH$_3$/$^{13}$NH$_4^+$. Influx showed significant stimulations with rising pH over the entire range (Fig. 33D) and followed clear Michaelis-Menten kinetics with rising [NH$_3$]$_{ext}$ (derived using the Henderson-Hasselbalch equation; Fig. 33E). By contrast, influx as a function of rising [NH$_4^+$]$_{ext}$ showed a declining pattern, particularly above 9 mM (Fig. 33F).
These effects were observed under both low (0.02 mM) and high (5 mM) external K+ concentrations, which were applied in context of the known regulation of NH$_3$/NH$_4^+$ fluxes by K+ (Szczerba et al. 2008; Balkos et al. 2010; ten Hoopen et al. 2010). Under low K+, where NH$_3$/NH$_4^+$ toxicity is most severe (Britto and Kronzucker 2002; Balkos et al. 2010), total influx plateaued at approximately 200 µmol g$^{-1}$ h$^{-1}$, the highest transmembrane flux of NH$_3$/NH$_4^+$ hitherto reported in any plant system. Under high K+, where relief from toxicity is observed (Britto and Kronzucker 2002), [NH$_3$]$_{ext}$-dependent influx was significantly lower, as apparent in the decrease in V$_{max}$ (from 204.8 ± 14.5 to 80.0 ± 4.5 µmol g$^{-1}$ h$^{-1}$ for low- and high-K+ plants, respectively). By contrast, no significant differences in K$_M$ were observed between K+ conditions (0.15 ± 0.05 versus 0.09 ± 0.03 mM for low- and high-K+ plants, respectively). The energetic consequences of increases in NH$_3$/NH$_4^+$ influx with pH were also tested using root respiration measurements. We found that, despite the much higher influx observed when pH was changed from 6.25 to 9.25, steady-state root O$_2$ consumption decreased by approximately 55% within 5 min of this change in low-K+, high-NH$_3$/NH$_4^+$ plants (Fig. 34). By contrast, no such changes were observed when nitrate (NO$_3^-$) was the sole N source.
Figure 33. NH₃ (not NH₄⁺) is the main permeating species in barley roots. A and B, Concentration dependence of influx (A) and ΔΔΨₘ (B) of NH₃/NH₄⁺ (red) and K⁺ (blue) in plants grown at 0.05 mM (NH₄)₂SO₄ and K₂SO₄. C, Data from A and B replotted to show relationship between ΔΔΨₘ and influx. D, Root NH₃/NH₄⁺ influx as a function of external pH. E and F, Data from D replotted to show dependence of NH₃/NH₄⁺ influx on NH₃ (E) or NH₄⁺ (F) concentrations, which were predicted from solution pH, according to the Henderson-Hasselbalch equation (pKa of NH₃/NH₄⁺ = 9.25). Area I represents pH 4.25 to 8.25; area II represents pH 8.25 to 9.25. Plants were grown at 5 mM (NH₄)₂SO₄ and either low (0.01 mM, red) or high (2.5 mM, blue) K₂SO₄. For all sections, error bars represent ± SE of the mean (n ≥ 3). Letters in D denote significantly different means (P < 0.05) as determined by a one-way ANOVA with Tukey’s post-hoc test.
Figure 34. Effect of 5-min exposure to elevated pH (pH 9.25) on root respiration in barley plants grown with 0.01 mM K$_2$SO$_4$ and 5 mM of either (NH$_4$)$_2$SO$_4$ or Ca(NO$_3$)$_2$. Red bar represents O$_2$ consumption, predicted if NH$_3$/NH$_4^+$ influx at pH 9.25 is comprised entirely of cationic NH$_4^+$ fluxes (see Fig. 33F). Letters denote significantly different means (P < 0.05) as determined by one-way ANOVA with Tukey’s post-hoc test.
As with influx, efflux of $^{13}\text{NH}_3/^{13}\text{NH}_4^+$ from pre-labeled roots was strongly stimulated by alkaline solution pH (and thus, higher external $[\text{NH}_3]$ to $[\text{NH}_4^+]$ ratios; Fig. S1A). In plants grown on high $\text{NH}_3/\text{NH}_4^+$ and low $K^+$, sudden (at 8 min; Fig. S1A, see arrow) upward shifts in external pH immediately and significantly stimulated $^{13}\text{NH}_3^{13}\text{NH}_4^+$ efflux, with greater stimulations observed at higher pH values. When tracer release was plotted as a function of the concomitant $\text{NH}_3/\text{NH}_4^+$ influx (measured at the identical pH shift), we observed a strong linear relationship between efflux and influx (Fig. S1B). Moreover, tracer release as a function of $[\text{NH}_3]_{\text{ext}}$ (which we suggest may be equivalent to cytosolic $[\text{NH}_3]$; see below) resulted in Michaelis-Menten kinetics similar to those seen with influx, having $K_M$ values ranging from 0.10 to 0.36 mM $[\text{NH}_3]$ (Fig. S1B, inset).

We sought to further characterize $^{13}\text{NH}_3^{13}\text{NH}_4^+$ efflux in intact roots under toxicity conditions (low $K^+$, high $\text{NH}_3/\text{NH}_4^+$) to identify the compartmental origin of tracer release (Britto and Kronzucker 2003; Coskun et al. 2010). In roots pre-labeled with tracer, $^{13}\text{NH}_3^{13}\text{NH}_4^+$ efflux was immediately suppressed by sudden (at 8 min; Fig. 35A, see arrow) exposure to 4 °C or upon withdrawal of external $\text{NH}_3/\text{NH}_4^+$, while an external pH shift to 9.25 (from 6.25) resulted in an immediate and sizable efflux stimulation. Importantly, these findings demonstrate that efflux analysis under toxic conditions captures physiological (i.e. transmembrane) events, not artifacts of apoplastic exchange (Coskun et al. 2010, 2013). Thus, compartmental analysis by tracer efflux could be applied (Lee and Clarkson 1986; Kronzucker et al. 1997; Britto and Kronzucker 2003), revealing efflux to influx ratios of approximately 80% and extremely rapid rates of both unidirectional fluxes characteristic of futile cycling (Britto et al. 2001b).

Further evidence for the intracellular origin of effluxed tracer was seen in a silver (Ag$^+$)-induced stimulation of $^{13}\text{NH}_3^{13}\text{NH}_4^+$ release (Fig. 35B). We have previously shown that sudden exposure to Ag$^+$ causes extensive damage to both major membrane systems (plasmalemma and tonoplast) in barley roots (Coskun et al. 2012). By contrast, we observed no effect of mercury (Hg$^{2+}$) application on tracer release (Fig. 35C), suggesting a lack of membrane disintegrity occurring. Importantly, this qualifies the use of Hg$^{2+}$ as a potential inhibitor of AQPs (see below). With respect to the Ag$^+$-induced stimulation in tracer efflux, this effect allowed for quantification of released substrate (in terms of µmol g$^{-1}$ root fresh weight) via integration of the $^{13}\text{N}$ loss and estimated intracellular specific activity, as shown previously (Coskun et al. 2012; see Chapter 5). The chemical quantity of $\text{NH}_3/\text{NH}_4^+$ released during Ag$^+$ application (64 ± 3 µmol g$^{-1}$) was very
similar to that of total root-tissue NH$_3$/NH$_4^+$ content under control conditions, as measured by chemical (orthophthalaldehyde [OPA]) analysis (63.8 ± 2.3 µmol g$^{-1}$; Fig. 35D). We should note that, although efflux still proceeded after termination of the Ag$^+$ treatment (Fig. 35B), the apparent premature curtailment of the treatment resulted in an underestimate of no more than approximately 1 µmol g$^{-1}$, which was considered negligible. Tissue analysis (determined by OPA assay) revealed that approximately 70% of root NH$_3$/NH$_4^+$ was lost during Ag$^+$ exposure, demonstrating that the majority of cellular (i.e. both cytoplasmic and vacuolar) NH$_3$/NH$_4^+$ was released (Fig. 35D). By contrast, pH 9.25 and Hg$^{2+}$ resulted in no change in tissue NH$_3$/NH$_4^+$ content (Fig. 35D), despite the significant effects on both influx and efflux of the former (see above).

Lastly, to gain mechanistic insight into NH$_3$/NH$_4^+$ influx, the possible involvement of different types of membrane transporters in NH$_3$/NH$_4^+$ influx was tested by means of pharmacological profiling, in low-K$^+$, high-NH$_3$/NH$_4^+$ plants (Fig. 36). Hg$^{2+}$, a well-known blocker of AQP activity, was applied with significant effect (36% inhibition at pH 6.25), while further support for AQP involvement was observed with treatments known to induce intracellular acidosis, which can cause closure of AQPs via protonation of conserved His residues on the cytoplasmic side (Tournaire-Roux et al. 2003; Törnroth-Horsefield et al. 2006; Ehlert et al. 2009). Hydrogen peroxide (H$_2$O$_2$) and propionic acid (PA) were two such effective treatments (30% and 54% inhibition relative to control, respectively). N$_2$ treatment, however, was not as effective, despite its efficacy in other systems (Tournaire-Roux et al. 2003). Note that these acidifying treatments were only effective at lower external pH (pH 5.25). Also, Hg$^{2+}$ could not be tested at high pH (pH 9.25) due to hydroxide precipitation (Schuster 1991). Other significant inhibitors of NH$_3$/NH$_4^+$ influx at pH 6.25 included Cs$^+$ < K$^+$ < La$^{3+}$ < Zn$^{2+}$ << 4 °C. The highest influx, seen at pH 9.25, was also suppressible at 4 °C by approximately 44%.
Figure 35. Characterization of NH$_3$/NH$_4^+$ efflux from roots of intact barley seedlings. A, Effect of sudden (at $t = 8$ min; see arrow) exposure to either pH 9.25, 4°C, or N withdrawal from the external medium. Each point represents the mean of three to seven replicates (SE of the mean <15% of the mean). B, Effect of sudden (at $t = 15$ min; see arrow) exposure to 500 µM Ag$^+$.

Integration range of tracer release due to Ag$^+$ and results of integration are given in shaded area. Each point represents the mean of four replicates (SE of the mean <15% of the mean). C, Lack of effect of 500 µM Hg$^{2+}$ application (at $t = 15.5$ min; see arrow) on root $^{13}$NH$_3$/NH$_4^+$ efflux. Each point represents the mean of three replicates (SE of the mean <15% of the mean). D, Root NH$_4^+$ content measured using OPA and its effect due to 15-min exposure to pH 9.25, 500 µM Hg$^{2+}$, or 500 µM Ag$^+$. Each bar represents mean ± SE of the mean (n ≥6). Asterisks denote significant difference from control (P ≤0.001) as determined by one-way ANOVA with Dunnett’s post-hoc test. In all sections, plants were grown at 5 mM (NH$_4$)$_2$SO$_4$ and 0.01 mM K$_2$SO$_4$.
**Figure 36.** Pharmacological profile of NH$_3$/NH$_4^+$ influx into roots of barley seedlings at varying external pH. Ionic inhibitors were applied as chloride salts, except for K$^+$ (applied as K$_2$SO$_4$). Influx at pH 9.25 corresponds to y-axis on the right. Each bar represents mean ± SE of the mean (n ≥5). Asterisks denote significantly different means (**, P ≤0.01; $$$, P ≤0.001) from respective control, as determined by one-way ANOVA with Dunnett’s post-hoc test (at pH 6.25 and 5.25) or Student’s $t$ test (at pH 9.25). Plants were grown as in Figure 35.
6.1.4 Discussion

This study critically reexamines the nature of futile transmembrane NH₃/NH₄⁺ cycling in barley roots, a phenomenon with ties to NH₄⁺ toxicity in a wide range of higher plants (Feng et al. 1994; Britto et al. 2001b; Britto and Kronzucker 2002; Chen et al. 2013). We have addressed the fundamental question of which species of the conjugate pair (NH₃ or NH₄⁺) is transported in the futile cycle to thus enable insight into mechanisms of transport, compartmentation, and toxicity of NH₃/NH₄⁺.

The lack of agreement between ΔΔΨₘ and changes in NH₃/NH₄⁺ influx, in contrast to K⁺, suggests that, above a small baseline cationic NH₄⁺ flux no higher than that of K⁺ (<5 µmol g⁻¹ h⁻¹; Fig. 33A), electroneutral NH₃ transport accounts for the observed rapid rates of ¹³N transport in intact barley roots (Fig. 33A–C). While previous tracer studies have also demonstrated that NH₃/NH₄⁺ fluxes exceed those of K⁺ at equimolar concentrations (Scherer et al. 1984; Vale et al. 1988b; Wang et al. 1996), none have provided parallel membrane potential measurements. A comparison between fluxes and ΔΔΨₘ, however, is of great utility in gauging the relative apportionment of NH₃ and NH₄⁺ fluxes, as we show here.

Because such rapid NH₃ fluxes in planta are simply without precedent, additional investigation was called for. Further evidence in support of NH₃ uptake was seen in the Michaelis-Menten character of the [NH₃]ₘₐₓ-dependent influx isotherms (Fig. 33E). By contrast, NH₄⁺ influx was seen to decline with rising [NH₄⁺]ₘₐₓ (Fig. 33F), ruling out a sizeable contribution from that N species. We should note, however, that because these isotherms were obtained using changes in external pH, there may be pH-specific and/or NH₄⁺-specific effects on transport. Such effects require examination, although they are inherently difficult to ascertain because pH and [NH₃] to [NH₄⁺] ratios are inextricably linked. It should also be noted that in a study on rice, Wang et al. (1993b) observed a decline in ¹³NH₃/¹³NH₄⁺ influx with rising pH at 10 mM [NH₃/NH₄⁺]ₘₐₓ. However, the fluxes in their study were much lower than in this study and also were determined in an NH₄⁺-tolerant species. Further investigation is necessary to determine whether this is a part of the strategy by which a plant may achieve tolerance to this N source.

We also provide evidence for NH₃ (but not NH₄⁺) efflux under toxic (low-K⁺, high-NH₃/NH₄⁺) conditions. Firstly, the trans-inhibition and -stimulation of efflux in response to changes in NH₃ provision (by substrate withdrawal and pH 9.2, respectively; Fig. 35A) suggests that NH₃ efflux
is highly dependent on NH₃ influx (see also the linear dependence of the fluxes; Fig. S1B), which is consistent with observations that efflux to influx ratios increase with rising influx (Wang et al. 1993a; Britto et al. 2002; Britto and Kronzucker 2006). Such trans-inhibition and -stimulation of efflux have previously been shown in barley (Britto and Kronzucker 2003) and in the mammalian literature, specifically for amino acids (White and Christensen 1982; Sweiry et al. 1991). In the latter case, trans-stimulation of efflux has been attributed to a large counterflow through a single transporter mediating bidirectional fluxes and, as such, could in large part explain the futile NH₃ cycling in this paper. Further evidence that the ¹³N efflux trace represents ¹³NH₃ and not ¹³NH₄⁺ is found in its saturating response to [NH₃]ₜ (Fig. S1B, inset), which resembles that of influx (Fig. 33E). Kₘ values for efflux, which were comparable to those for influx (ranging between 0.10–0.36 mM NH₃), suggests a similar, if not identical, mechanism of NH₃ transport for the two fluxes. It is not clear why the efflux step should respond so readily to changes in external NH₃, when substrate binding to an efflux transporter must take place intracellularly. Intriguingly, it may be that NH₃ transport responds to [NH₃]ₜ in a manner that leads to a rapid equalization between NH₃ pools on either side of the plasma membrane, and thus NH₃ efflux kinetics are in fact directly responding to cytosolic [NH₃] and only indirectly to [NH₃]ₜ. NH₃ may shuttle rapidly among multiple cellular compartments, establishing similar equilibrium concentrations in each, where membrane permeabilities permit (see below).

The Michaelis-Menten analyses under low-K⁺, high-NH₃/NH₄⁺ conditions (see above; Fig. 33E) revealed a Vₘₐₓ of about 200 µmol g⁻¹ h⁻¹ for NH₃ influx, the highest bona-fide transmembrane flux hitherto reported in any plant system. Such rapid fluxes are orders-of-magnitude higher than typical fluxes of mineral (ionic) nutrients (Britto and Kronzucker 2006). Although fluxes of sodium (Na⁺) under toxic (saline) conditions have been reported to reach or exceed such values (Lazof and Cheeseman 1986; Essah et al. 2003; Malagoli et al. 2008), the validity of these fluxes have recently come into question, particularly with respect to their unrealistic energetic requirements (Britto and Kronzucker 2009; Kronzucker and Britto 2011); moreover, such fluxes are generally reported at much higher external substrate concentrations (typically, 100 mM or higher). On the other hand, such energetic limitations do not apply to the passive electroneutral fluxes of NH₃. In fact, root O₂ consumption was found to decline under such conditions (i.e. pH 9.25; Fig. 34), a result that further discounts NH₄⁺-specific futile cycling, which is predicted to involve a thermodynamically active efflux (Britto et al. 2001b). Figure 34 highlights, in red, the
theoretical increase in O$_2$ consumption necessary to power an active efflux mechanism of NH$_4^+$ when fluxes are as high as 200 µmol g$^{-1}$ h$^{-1}$ based on current models of ion transport and O$_2$ consumption (Poorter et al. 1991; Kurimoto et al. 2004; Britto and Kronzucker 2009). This large energy deficit is consistent with the idea that futile cycling is primarily of the conjugate base, NH$_3$. However, unlike with the previously proposed NH$_4^+$ cycling, the term futile here does not refer to an energy-dissipating process (e.g. Amthor 2000), but more generally to the lack of apparent functional utility in the NH$_3$ cycle. The fact that the pH shift did not affect root O$_2$ consumption in NO$_3^-$-grown plants shows an N-source specificity of this effect that will require further investigation to explain.

These results have important consequences for the compartmentation and toxicity of NH$_3$/NH$_4^+$. Compartmental analyses with $^{15}$NH$_3$/$^{15}$NH$_4^+$ in roots and shoots of plants grown under toxic (high-NH$_3$/NH$_4^+$) conditions generally yield extremely high “pool sizes” of many hundred millimolar (Britto et al. 2001b, 2002), leading to the speculation that the entire cell, not simply the cytosol, acts as a single compartment of tracer origin (Britto and Kronzucker 2003; Balkos et al. 2010). This study provides the first evidence in support of this “whole-cell” hypothesis. Because a high-capacity, thermodynamically passive NH$_3$ transport can account for futile cycling, it is feasible that NH$_3$ rapidly equilibrates across intracellular membranes and among cellular compartments, particularly the vacuole and cytosol (Fig. 37). How such rapid unidirectional fluxes can persist given the apparent lack of an NH$_3$ concentration gradient across cellular compartments is an interesting question and can be most simply explained by passive diffusion through high-capacity membrane channels such as AQPs (see below). The NH$_3$ equilibration across cellular compartments can explain why tracer accumulation (measured as counts retained in tissue or released with Ag$^+$ application; Fig. 35B) closely agreed with that of chemical (OPA) analyses measuring tissue NH$_3$/NH$_4^+$ content (Fig. 35D). Thus, the NH$_4^+$ content within each compartment may be ultimately determined by NH$_3$ permeation and compartmental pH, as illustrated in Figure 37. In this revised model of futile cellular N cycling, the 0.5 to 1.5 mM range of cytosolic [NH$_4^+$] agrees well with measured values from studies using methods such as ion-selective microelectrodes and NMR (Lee and Ratcliffe 1991; Wells and Miller 2000). Importantly, the model reveals that a hyperaccumulation of NH$_4^+$ in the vacuole would ultimately exist (Fig. 37), due to vacuolar acid trapping, and could explain the frequently observed suppressions in cationic nutrients, notably K$^+$ (but also Ca$^{2+}$ and Mg$^{2+}$; Barker et al.
1967; Van Beusichem et al. 1988; Lang and Kaiser 1994; Kronzucker et al. 2003), which may ultimately be the major cause of $\text{NH}_3$/\text{NH}_4\text{+} toxicity in higher plants.

It is likely that the rapid NH$_3$ cycling reported here is mediated by AQPs (Fig. 36), which have high transport capacity and are known to conduct NH$_3$ fluxes (Jahn et al. 2004; Holm et al. 2005; Loqué et al. 2005; Saparov et al. 2007; Hove and Bhave 2011). Kozono et al. (2002) estimated the rate of water transport through AQP1 to be $3 \times 10^9$ molecules per subunit per second, roughly 30-fold higher than K\textsuperscript{+} transport via the potassium crystallographically-sited activation (KcsA) channel, which is among the fastest ion channels (Morais-Cabral et al. 2001). AQP involvement is suggested by the pharmacological profiling of NH$_3$ influx in our study, particularly in the effect of Hg$^{2+}$, a classic AQP inhibitor (Fig. 36). Importantly, unlike with Ag$^+$ (Fig. 35B), Hg$^{2+}$ showed no sign of causing membrane damage in our system (as manifest in lack of efflux stimulation or tissue content losses; Fig. 35C and D; compare with Coskun et al. 2012; Chapter 5). The strong suppressions of influx by H$_2$O$_2$ and PA also support AQP involvement (Fig. 36; Tournaire-Roux et al. 2003; Törnroth-Horsefield et al. 2006; Ehlert et al. 2009). It is worth highlighting here, however, that pharmacological profiling, like any method, is not without its caveats. The lack of specificity of several blockers/chemical treatments (Coskun et al. 2013c), as well as the need to employ relatively high concentrations at times, can potentially have secondary effects. This by no means invalidates the use of pharmacology, but highlights the importance of a diversity of experimental approaches. Future experiments with AQP antisense/knockout lines (Martre et al. 2002; Javot et al. 2003), particularly for AQPs already shown to mediate NH$_3$ fluxes (Jahn et al. 2004; Holm et al. 2005; Loqué et al. 2005), could help further elucidate their involvement in futile NH$_3$ cycling. In addition, such mutant analyses could provide a critical test of the “whole-cell distribution” hypothesis for NH$_3$ presented above (Fig. 37).

We end by highlighting the suppression of $V_{\text{max}}$ for NH$_3$ influx by high-plant K\textsuperscript{+} status, while $K_M$ remains unaffected (Fig. 33E). It appears that K\textsuperscript{+} status has no effect on the substrate affinity of NH$_3$ transporters but regulates NH$_3$ influx by other means. One such mechanism might involve the modulation of AQP activity, which may well be expected, because, in plants, K\textsuperscript{+} acquisition is the chief means of establishing osmotic balance and cell turgor (Britto and Kronzucker 2008; Grzebisz et al. 2013). The effects of K\textsuperscript{+} in the short-term suppression of NH$_3$/NH$_4\text{+}$ influx and efflux (Szczerba et al. 2008; Balkos et al. 2010; see also Fig. 36) also suggest a posttranslational
regulation of NH$_3$ transporters by K$^+$. Such a mechanism may explain the agriculturally important alleviation of NH$_3$/NH$_4^+$ toxicity in higher plants by K$^+$ (Barker et al. 1967; Szczerba et al. 2008; Balkos et al. 2010) and thus pave the way for future studies.
**Figure 37.** Revised model of futile transmembrane \( \text{NH}_3/\text{NH}_4^+ \) cycling in root cells of higher plants. Uncharged \( \text{NH}_3 \) rapidly equilibrates across both major membrane systems (plasmalemma and tonoplast) and is likely mediated by AQPs specific to each system. A relatively minor channel/carrier-mediated flux of \( \text{NH}_4^+ \) may also occur across both membrane systems. \( \text{NH}_4^+ \) concentrations are a function of \( \text{NH}_3 \) equilibration and compartment pH (Roberts et al. 1982; Walker et al. 1996a; Kosegarten et al. 1997; 1999). PIP, Plasmalemma intrinsic protein; TIP, tonoplast-intrinsic protein; AMT, ammonium transporter; Kir, \( \text{K}^+ \) inward rectifier; NSCC, nonselective cation channel.
Chapter 7
Conclusions and Future Directions

This work highlights the physiological involvement of several important membrane channels to plant mineral nutrition (i.e. K⁺ influx and efflux) and ion toxicity (i.e. K⁺ influx and efflux under Na⁺ and NH₄⁺ toxicity, as well as NH₃/NH₄⁺ transport under ammonium toxicity).

Subchapter 3.1 demonstrated the physiological mechanisms and regulations of K⁺ release in roots of intact barley seedlings under typical, non-stressed conditions, as well as in response to sudden nutritional and stress-induced shifts. We showed that bona fide transmembrane efflux of K⁺ across the plasma membrane is only observed at low [K⁺]₆ₓ (i.e. <1 mM), and is indeed channel-mediated (likely via an unidentified outward-rectifying Shaker-like channel). Above this concentration (i.e. ≥1 mM [K⁺]₆ₓ), a thermodynamic shift in K⁺ transport is observed (i.e. from active to passive K⁺ influx; Britto and Kronzucker 2008). Interestingly, however, efflux under these conditions does not show a shift in mechanism (i.e. from passive to active transport) per se, but rather, transmembrane K⁺ efflux ceases altogether (i.e. there is no evidence for thermodynamically active, metabolically dependent, K⁺ efflux), and what is observed is simply release of ⁴²K⁺ from the extracellular matrix (apoplast). Moreover, important insights were gained in the response of K⁺ efflux to shifts in N-source provision, i.e. significant stimulations with elevated [NH₄⁺]₆ₓ (under both low and high [K⁺]₆ₓ, likely the response of depolarization-activated outward-rectifying K⁺ channels), and inhibitions by elevated external [NO₃⁻] (only at low [K⁺]₆ₓ). In contrast, elevated external [Na⁺] does not seem to affect K⁺ efflux, until such point that osmotically challenging concentrations (i.e. ≥100 mM) compromise membrane stability and thus bring about the release of cellular contents (including K⁺; see also Britto et al. 2010). Importantly, this work reveals the limitations of Compartmental Analysis by Tracer Efflux (CATE), used to quantify cellular pool sizes (e.g. [K⁺]ₗₚ) and half-times of exchange (₇/₂) by analyzing efflux kinetics (Lee and Clarkson 1986; Siddiqi et al. 1991; Kronzucker et al. 1995). By demonstrating that K⁺ efflux ceases altogether ≥1 mM [K⁺]₆ₓ, we show that CATE, while a powerful tool under some conditions, cannot be utilized at high [K⁺]₆ₓ. Thus, many earlier investigations quantifying [K⁺]ₗₚ and K⁺-exchange kinetics under low-affinity conditions
must be re-examined in this light (e.g. Szczerba et al. 2006), as well as many Na\textsuperscript{+} CATE experiments under similar conditions (Britto and Kronzucker 2015).

This work also explored the involvement of outward-rectifying K\textsuperscript{+} channels under short-term Na\textsuperscript{+} stress (Subchapter 3.2) and concluded that channels are not involved under these conditions in rice, contrary to standing models (Shabala et al. 2006), but in agreement with earlier analyses in barley from our laboratory (Britto et al. 2010; Coskun et al. 2010; Subchapter 3.1).

Importantly, Na\textsuperscript{+}-induced K\textsuperscript{+} efflux was found not to correlate with cultivar performance under long-term Na\textsuperscript{+} stress, also in contradiction to standing models (Chen et al. 2005; 2008; Cuin et al. 2008). It was concluded that the best measure for salt tolerance, at least for rice, remains the ability to limit the accumulation of Na\textsuperscript{+} in shoots (Yeo and Flowers 1982; 1983).

Chapter 4 highlighted the complexity of K\textsuperscript{+} uptake in roots of higher plants and the roles that K\textsuperscript{+} channels play (and don’t play) under various nutritional conditions. Subchapter 4.1 demonstrated the limits of the Arabidopsis model of K\textsuperscript{+} acquisition in terms of its extrapolation to cereals: unlike in Arabidopsis, K\textsuperscript{+} channels (e.g. HvAKT1) cannot operate under low (0.02 mM)-K\textsuperscript{+}, high (10 mM)-NH\textsubscript{4}\textsuperscript{+} conditions in barley; rather, influx is thermodynamically active and although highly suppressed by NH\textsubscript{4}\textsuperscript{+}, likely mediated by residual HvHAK1 activity. Moreover, no evidence was found for a discernable contribution of NSCCs to K\textsuperscript{+} uptake in planta under a wide range of conditions (high- and low-affinity, stressed and non-stressed), which has important implications for currently accepted Na\textsuperscript{+}-toxicity models, as it indicates that NSCCs may not be significantly involved in Na\textsuperscript{+} transport either (Kronzucker and Britto 2011; cf. Davenport and Tester 2000; Demidchik et al. 2002; Demidchik and Maathuis 2007).

Furthermore, some of the highest bona fide channel-mediated ion fluxes in plant roots were measured (up to 36 µmol K\textsuperscript{+} g\textsuperscript{-1} h\textsuperscript{-1}) by investigating the effects of NH\textsubscript{4}\textsuperscript{+} withdrawal in NH\textsubscript{4}\textsuperscript{+}-grown plants and the stimulatory effects of counterions, such as Cl\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}, under high [K\textsuperscript{+}]\textsubscript{ext} (see also Subchapter 4.2).

By contrast, Subchapter 4.3 demonstrated that channel-mediated K\textsuperscript{+} influxes in the low-affinity (saline) range were quite modest (i.e. no higher than 15 µmol g\textsuperscript{-1} h\textsuperscript{-1}), despite the observation of apparent influxes that can be extremely high (2-3 orders of magnitude higher). These apparent fluxes, as measured by radiotracer and which showed linear dependences on [K\textsuperscript{+}]\textsubscript{ext}, were demonstrated to originate from the root apoplast, despite attempts to clear the extracellular matrix of radiotracer. This hitherto overlooked, yet significant, contribution of apoplastic fluxes
to ion-flux determinations has far-reaching implications, especially for Na\(^+\) fluxes under salinity stress. According to currently accepted models, root Na\(^+\) influx shows linear dependences with external Na\(^+\) supply, can be extremely rapid (*e.g.* up to 600 \(\mu\)mol g\(^{-1}\) h\(^{-1}\); Lazof and Cheeseman 1986; Essah et al. 2003; Horie et al. 2007; Kronzucker and Britto 2011), and is channel (NSCC)-mediated (Demidchik et al. 2002; Essah et al. 2003; Munns and Tester 2008). Given that standing models of Na\(^+\) transport in salt-stressed roots may be afflicted by an apoplastic artefact, much more work is needed to adequately appraise the role of the apoplast in flux measurements in the saline range. One such step is to take advantage of the available means to alter the composition of the apoplast, and monitor the effects on ion fluxes. For example, Ranathunge et al. (2016) have shown that elevated NH\(_4^+\) supply in rice seedlings can increase the suberin and lignin composition of roots, thereby increasing apoplastic barriers to solute permeability (Schreiber et al. 1999). How this might affect low-affinity fluxes of ions like Na\(^+\) and K\(^+\) would be worth exploring, although one must keep in mind the potential compounding stresses of Na\(^+\) and NH\(_4^+\) (Speer and Kaiser 1994; Speer et al. 1994). Another nutritional modification of the apoplast involves the application of silicon (Si). Si has been shown to increase Casparian band formation, as well as provide a mechanical barrier to apoplastic transport via biosilicification (*i.e.* amorphous silica deposition; Fleck et al. 2011; Exley 2015; Guerriero et al. 2016). Early investigations in our laboratory have shown that Si supply does not affect the unidirectional fluxes of Na\(^+\) in roots of salt-stressed rice (but significantly suppresses root-to-shoot translocation); however, more investigation is required here. Another way to alter the apoplast involves stagnant deoxygenation of roots (*e.g.* growth in 0.1\% agar solution, which has been shown to mimic waterlogging conditions) which enhances root suberization and lignification in rice (Ranathunge et al. 2011). Mutants also exist that express apoplastic modifications, such as Arabidopsis genotypes with lower suberin content (*e.g.* cyp86 \(a1\) and cyp86 \(b1\); Hoefer et al. 2008; Compagnon et al. 2009; Molina et al. 2009; Franke et al. 2012) and those with enhanced suberin content (*e.g.* kcs1; Todd et al. 1999; Franke et al. 2009). Recent investigations in our laboratory with these mutants have shown that, indeed, Na\(^+\) fluxes can be negatively correlated with suberin production; Schulze 2014). Moreover, a clade of ATP-binding cassette (ABC) transporters, AtABCG2, AtABCG6, and AtABCG10 in Arabidopsis (Yadav et al. 2014), and OsABCG5 in rice (Shiono et al. 2014), have been shown to be required for effective suberin synthesis in roots. Thus, using radiotracers for ions of interest (\(^{24}\)Na\(^+\) and \(^{42}\)K\(^+\)), as well as tracers specific for the apoplast (*e.g.* PTS, periodic acid, and berberine; Peterson et al. 1981; Yeo et al.
we can measure apoplastic transport under these various growth conditions and genotypes to better understand this complex tissue and its role in ion transport under salinity stress.

The role of the BUS (i.e. the K⁺-uptake “back-up system” revealed in athak5 atakt1 double-knock-out mutants), particularly in low-affinity K⁺ transport (but perhaps also in low-affinity Na⁺ transport), will also require much more investigation. Early indications from the laboratory of F. Rubio suggest that this system may be a NSCC, possibly of the cyclic-nucleotide gated (CNGC) variety (Caballero et al. 2012). However, in contrast to the findings of Caballero et al. (2012), our experiments with athak5 atakt1 mutants showed no sensitivity of K⁺ influx to Ca²⁺ or Ba²⁺ (Subchapter 4.1), although further testing of the inhibitors they used, including Mg²⁺, La³⁺, and the cyclic nucleotide N6,2'-O-dibutyryladenosine 3'-5'-cyclic monophosphate, should be conducted. Also, the nature of the “anion effect” (i.e. Cl⁻- and NO₃⁻-induced stimulation) in low-affinity K⁺ transport should be investigated more thoroughly (Chapter 4; Kochian et al. 1985). Cation-chloride cotransporters (CCCs), symporters that simultaneously, and electroneutrally, transport K⁺ (and/or Na⁺) along with Cl⁻ (Colmenero-Flores et al. 2007), are another obvious target of investigation. Pharmacological testing of CCCs, such as with bumetanide, could be conducted to test for CCC contribution. Moreover, mutants in Arabidopsis AtCCC1 (Colmenero-Flores et al. 2007) and rice OsCCC1 (Kong et al. 2011; Chen et al. 2016) could be investigated for anion-sensitive low-affinity K⁺ (and Na⁺) fluxes. However, the fact that NO₃⁻ is also capable of stimulating low-affinity K⁺ transport suggests that another mechanism may be at play. The coupling of K⁺ and anion transport has typically been postulated as a potential mechanism (Kochian et al. 1985); however, a mechanism for this coupling has yet to be resolved. Recently, a study showed that S-type anion channels SLAH3 and SLAC1 (which are both permeable to nitrate and chloride) in Arabidopsis guard cells physically interact and regulate K⁺ channels, albeit negatively (Zhang et al. 2016). Although this study showed that anion channels inhibit K⁺ channels in guard cells, the study raises the question whether cation-anion channel interactions may occur at the level of the root, and perhaps in a positive way.

Chapter 5 highlighted the importance of rigorous testing of pharmacological agents, such as the commonly used heavy-metal AQP inhibitors Hg²⁺, Ag⁺, and Au³⁺, which were found to have significant side effects on K⁺ transport and cellular membrane stability, including membrane destruction brought about by high (500 µM) concentrations of Au³⁺ and Hg²⁺, and a wide range
(5-500 µM) of Ag⁺ concentrations, rendering cells incapable of retaining critical constituents. Moreover, significant inhibition of K⁺ influx at lower (5 and 50 µM) concentrations of Au³⁺ and Hg²⁺ were observed, without noticeable effects on membrane stability. Interestingly, these side effects, particularly with Ag⁺, have been instrumental in the determination of cytosolic pool sizes, especially under conditions where traditional methods such as CATE cannot be used (see above). Taking advantage of this “Ag⁺-bullet” technique, detailed measurements of Na⁺ and K⁺ concentrations in the cytosol under Na⁺-stress conditions are currently underway in our laboratory. Interestingly, contrary to previous estimates based on kinetic flux and compartmentation analyses (Kronzucker et al. 2013), cytosolic Na⁺ estimates based on the “Ag⁺-bullet” technique suggest rather modest concentrations, no higher than 30 mM, in good agreement with ion-selective microelectrode recordings (Carden et al. 2003). By comparison, early indications suggest that the cytosolic K⁺ pool sizes under Na⁺ stress significantly decline from the well documented homeostatic levels of ~100 mM (Walker et al. 1996a; Britto and Kronzucker 2008), perhaps revealing an important mechanistic link to Na⁺ toxicity.

Chapter 6 demonstrated that low-affinity NH₃/NH₄⁺ fluxes in roots of barley seedlings under ammonium toxicity were predominately of the gaseous species NH₃, rather than ionic NH₄⁺ (fluxes of the latter were no higher than K⁺ fluxes under comparable conditions, i.e. <5 µmol g⁻¹ h⁻¹). Compared to some of the more rapid K⁺ fluxes observed (e.g. see Chapter 3), NH₃ fluxes were found to be ~5.5-fold higher (i.e. exceeding 200 µmol g⁻¹ h⁻¹), and were indeed some of the highest bona fide transmembrane fluxes ever reported in planta. Keeping the lessons from Chapter 5 in mind, early pharmacological evidence from this study suggested that AQPs may mediate these NH₃ fluxes; however, more investigation is required. Future experiments should investigate NH₃ fluxes in planta for knock-out mutants of AQPs previously demonstrated to mediate NH₃ fluxes in heterologous expression systems (e.g. AtTIP2;1 and AtTIP2;3 in Arabidopsis; Loqué et al. 2005). Interestingly, a recent study on the novel crystal structure of AtTIP2;1 speculates that a flexible histidine residue in a water-filled side pore of the channel might be involved in the deprotonation of NH₄⁺, thereby possibly increasing the permeation of NH₃ (Kirscht et al. 2016). Taken together, we proposed a revised model (Fig. 37) of NH₃/NH₄⁺ transport and compartmentation under ammonium toxicity in root cells, whereby AQP-mediated NH₃ fluxes across both major membrane systems (i.e. plasmalemma and tonoplast) result in the rapid equilibration of NH₃ across the cytosol and vacuole. NH₃ equilibration and compartment
pH thus result in the ‘acid trapping’ and toxic accumulation of NH$_4^+$ in the vacuole, which may underlie the toxicity syndrome.

A promising route of future study based on the work from Chapter 6 involves the regulation of NH$_3$ fluxes by K$^+$. Elevated K$^+$ supply, both in the short and long term, can significantly inhibit NH$_3$/NH$_4^+$ fluxes under ammonium toxicity, as well as relieve the symptoms of ammonium stress (Britto and Kronzucker 2002; Szczera et al. 2008b; Balkos et al. 2010; Coskun et al. 2013b). Thus, future studies will test the hypothesis that K$^+$ can directly regulate AQP-mediated fluxes. In the Michaelis-Menten analyses of Chapter 6 (see Fig. 33E), elevated K$^+$ suppressed the $V_{\text{max}}$ of NH$_3$ influx, but did not alter the $K_M$, suggesting that K$^+$ may not have an effect on the substrate binding affinity of AQPs, but may regulate their activity by other means (perhaps via changes in conformation). Long-term (3-mo) studies in rice plants demonstrated a 127% increase in agronomic water-use efficiency (WUE$_a$) in high (1.5 mM)-K$^+$ relative to low (0.1 mM)-K$^+$ plants (Britto et al. 2014), suggesting a regulatory role for K$^+$ in water transport. Also, preliminary evidence from our laboratory suggests that water transport (transpiration measured gravimetrically, and root hydraulic conductance measured via the pressure chamber technique; Scholander et al. 1965; Tournaire-Roux et al. 2003) is indeed lower in high-K$^+$ relative to low-K$^+$ plants (data not shown), further suggesting AQP-mediated fluxes may be regulated by K$^+$ status. Future studies will also investigate the role of K$^+$ in regulating AQP-mediated water transport by measuring hydraulic conductance of root (cortical) cells by the cell pressure probe technique (Tomos and Leigh 1999; Javot et al. 2003) as well as osmotic water permeability ($P_f$) in heterologous expression systems (e.g. Xenopus oocytes) employing osmotic swelling assays (Maurel et al. 1995). Such studies will be vital not only to address the issue of ammonium toxicity by potentially regulating the toxic cellular accumulation of NH$_3$/NH$_4^+$, but also to improve plant WUE, especially in the context of agriculture, which currently consumers 80-90% of the fresh water available for human consumption worldwide (Hamdy et al. 2003; Hoekstra and Chapagain 2007).
References


Appendix I: Other Published Material

The following is a list of review and methods articles, along with their abstracts, of which I was the primary author, and which have been published in peer-reviewed journals over the course of my thesis project.


**Abstract:** Unidirectional influx and efflux of nutrients and toxicants, and their resultant net fluxes, are central to the nutrition and toxicology of plants. Radioisotope tracing is a major technique used to measure such fluxes, both within plants, and between plants and their environments. Flux data obtained with radiotracer protocols can help elucidate the capacity, mechanism, regulation, and energetics of transport systems for specific mineral nutrients or toxicants, and can provide insight into compartmentation and turnover rates of subcellular mineral and metabolite pools. Here, we describe two major radioisotope protocols used in plant biology: direct influx (DI) and compartmental analysis by tracer efflux (CATE). We focus on flux measurement of potassium (K\(^+\)) as a nutrient, and ammonia/ammonium (NH\(_3\)/NH\(_4^+\)) as a toxicant, in intact seedlings of the model species barley (*Hordeum vulgare* L.). These protocols can be readily adapted to other experimental systems (e.g. different species, excised plant material, and other mineral nutrients/toxicants). Advantages and limitations of these protocols are discussed.


**Abstract:** K\(^+\) channels are among the best-characterized classes of membrane protein in plants. Nevertheless, *in-planta* demonstrations of traits emerging from molecular characterizations have often been insufficient or lacking altogether. Such linkages are, however, critical to our basic understanding of plant nutrition and to addressing ‘real-world’ issues that are faced in environmental and agricultural settings. Here, we cover some of the recent advances in K\(^+\) acquisition with particular focus on voltage-gated K\(^+\)
channel functioning and regulation in roots, and highlight where linkages to \textit{in-planta} behaviour have been successfully made and, conversely, where such linkages are yet to be made.


\textbf{Abstract}: Nitrogen (N) and potassium (K) are the two most abundantly acquired mineral elements by plants, and their acquisition pathways interact in complex ways. Here, we review pivotal interactions with respect to root acquisition, storage, translocation and metabolism, between the K$^+$ ion and the two major N sources, ammonium (NH$_4^+$) and nitrate (NO$_3^-$). The intersections between N and K physiology are explored at a number of organizational levels, from molecular-genetic processes, to compartmentation, to whole plant physiology, and discussed in the context of both N-K cooperation and antagonism. Nutritional regulation and optimization of plant growth, yield, metabolism and water-use efficiency are also discussed.


\textbf{Abstract}: Carbon dioxide (CO$_2$) concentrations in the earth’s atmosphere are projected to rise from current levels near 400 ppm to over 700 ppm by the end of the 21st century. Projections over this time frame must take into account the increases in total net primary production (NPP) expected from terrestrial plants, which result from elevated CO$_2$ (eCO$_2$) and have the potential to mitigate the impact of anthropogenic CO$_2$ emissions. However, a growing body of evidence indicates that limitations in soil nutrients, particularly nitrogen (N), the soil nutrient most limiting to plant growth, may greatly constrain future carbon fixation. Here, we review recent studies about the relationships between soil N supply, plant N nutrition, and carbon fixation in higher plants under eCO$_2$, highlighting key discoveries made in the field, particularly from free-air CO$_2$
enrichment (FACE) technology, and relate these findings to physiological and ecological mechanisms.


**Abstract:** Although deemed a “non-essential” mineral nutrient, silicon (Si) is clearly beneficial to plant growth and development, particularly under stress conditions, including salinity and drought. Here, we review recent research on the physiological, biochemical, and molecular mechanisms underlying Si-induced alleviation of osmotic and ionic stresses associated with salinity and drought. We distinguish between changes observed in the apoplast (*i.e.* suberization, lignification, and silicification of the extracellular matrix; transpirational bypass flow of solutes and water), and those of the symplast (*i.e.* transmembrane transport of solutes and water; gene expression; oxidative stress; metabolism), and discuss these features in the context of Si biogeochemistry and bioavailability in agricultural soils, evaluating the prospect of using Si fertilization to increase crop yield and stress tolerance under salinity and drought conditions.