PHYSIOLOGY OF POTASSIUM NUTRITION IN CEREALS: FLUXES, COMPARTMENTATION, AND IONIC INTERACTIONS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Botany
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Doctor of Philosophy

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Abstract

Potassium (K\(^+\)) is an essential nutrient and the most abundant cation in plant cells. Plants possess two transport systems for K\(^+\) acquisition: a high-affinity system (HATS), operating at external K\(^+\) concentrations ([K\(^+\)]\(_{\text{ext}}\)) below 1 mM, and showing reduced transport activity in the presence of ammonium (NH\(_4^+\)); and, a low-affinity system (LATS), operating at [K\(^+\)]\(_{\text{ext}}\) above 1 mM, that is not affected by NH\(_4^+\). K\(^+\) transport and compartmentation were investigated in barley (Hordeum vulgare L.) and rice (Oryza sativa L.) using the non-invasive technique of compartmental analysis by tracer efflux (CATE), to simultaneously determine unidirectional membrane fluxes, ion concentrations, and exchange characteristics in subcellular compartments. These studies revealed striking differences in unidirectional K\(^+\) fluxes between HATS and LATS. It was found that flux measurements, using traditional direct influx (DI) protocols, accurately represented HATS influx, but underestimated LATS influx by as much as seven-fold. In both barley and rice, LATS K\(^+\) fluxes were found to undergo rapid, futile cycling, with the ratio of efflux:influx 3 to 5 times greater, and the cytosolic exchange rate 2 to 3 times faster than under HATS. Based upon plasma-membrane electrical potential measurements, efflux was found
to be active under LATS conditions. LATS-mediated conditions for K$^+$ were found to provide relief from NH$_4^+$ toxicity in barley by immediately reducing NH$_4^+$ influx by more than 50%, and significantly reducing NH$_4^+$ futile cycling. Employing the K$^+$ channel inhibitors cesium, lanthanum, and tetraethylammonium, NH$_4^+$ was shown to have both K$^+$-sensitive and – insensitive influx pathways at high [NH$_4^+$]$_{ext}$. Based on current models of flux energetics, the combined uptake of K$^+$ and NH$_4^+$ was found to utilize 60% of root oxygen consumption. Barley and rice both showed signs of NH$_4^+$ toxicity at low [K$^+$]$_{ext}$, but rice recovered at much lower [K$^+$]$_{ext}$, suggesting a crucial role of K$^+$ in the NH$_4^+$-tolerance of rice. These experiments address fundamental aspects of K$^+$ fluxes, and help provide a physiological framework for future studies of K$^+$ transport and mineral nutrition.
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>GYGD</td>
<td>Amino acid sequence of glycine-tyrosine-glycine-aspartate</td>
</tr>
<tr>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>Ammonium ion</td>
</tr>
<tr>
<td>Ba\textsuperscript{2+}</td>
<td>Barium ion</td>
</tr>
<tr>
<td>Cd\textsuperscript{2+}</td>
<td>Cadmium ion</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Cs\textsuperscript{+}</td>
<td>Cesium ion</td>
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<tr>
<td>Cl\textsuperscript{−}</td>
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</tr>
<tr>
<td>CATE</td>
<td>Compartmental analysis by tracer efflux</td>
</tr>
<tr>
<td>CNGCs</td>
<td>Cyclic-nucleotide gated channels</td>
</tr>
<tr>
<td>DI</td>
<td>Direct influx</td>
</tr>
<tr>
<td>Gd\textsuperscript{3+}</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>K\textsubscript{M}</td>
<td>Half-saturation concentration of reaction</td>
</tr>
<tr>
<td>HATS</td>
<td>High-affinity transport system</td>
</tr>
<tr>
<td>La\textsuperscript{3+}</td>
<td>Lanthanum ion</td>
</tr>
<tr>
<td>LATS</td>
<td>Low-affinity transport system</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>Mn\textsuperscript{2+}</td>
<td>Manganese ion</td>
</tr>
<tr>
<td>V\textsubscript{max}</td>
<td>Maximal velocity of reaction</td>
</tr>
</tbody>
</table>
NO$_3^-$  Nitrate ion
PO$_4^{3-}$, P$_i$  Phosphate ion (inorganic phosphate)
K$^+$  Potassium ion
H$^+$  Proton
Rb$^+$  Rubidium ion
Na$^+$  Sodium ion
SA  Specific activity
SCATE  Subsampling-based compartmental analysis by tracer efflux
SO$_4^{2-}$  Sulfate ion
TPK  Tandem-pore K$^+$ channels
$\Delta\Psi$  Trans-plasma-membrane electrical potential
TEA  Tetraethylammonium ion
Zn$^{2+}$  Zinc ion
[ion]$_{\text{cyt}}$  Cytosolic ion concentration
[ion]$_{\text{ext}}$  External ion concentration
[ion]$_{\text{vac}}$  Vacuolar ion concentration

**Genetic annotation**

Uppercase  Referring to the protein e.g. AKT
Uppercase italicized  Referring to the gene e.g. AKT
Lowercase italicized  Referring to a mutant form of the gene e.g. akt
CHAPTER 1:

General Introduction
Functions of $K^+$

Potassium is a vital nutrient for plant growth and development. Plants are able to accumulate substantial tissue contents of $K^+$, with some estimates of $K^+$ comprising 10% of plant dry weight, while, subcellularly, $K^+$ is the most abundant cation in plant cells (Leigh and Wyn Jones, 1984; Véry and Sentenac, 2003). Soil solution $K^+$ concentrations can vary widely, with estimates ranging from 0.1 to 10 mM (Reisenauer 1966; Hawkesford and Miller 2004). Despite the occurrence of high soil $K^+$ concentrations, particularly in agricultural environments, far more is known about mechanisms mediating $K^+$ transport at low external concentrations (Gierth and Mäser, 2007; Lebaudy et al., 2007). As the growth rate of the world’s population outstrips that of grain production (Khush, 2005), an understanding of the pathways mediating, and driving forces affecting $K^+$ transport becomes urgently necessary. Such examinations must not only focus on the cellular components of $K^+$ transport, but include whole-plant investigations that can lead to potential improvements of crop yields beyond current limitations.

Functional studies of $K^+$ have determined two broad categories describing the role of $K^+$, either as biophysical or as biochemical, although these roles tend to overlap. $K^+$ pools can be found throughout the plant cell, with considerable accumulation in the cytosolic and vacuolar compartments. $[K^+]_{\text{cyt}}$ has been described as being homeostatically set at approximately 100 mM, while $[K^+]_{\text{vac}}$ has been found to vary between 10 to 500 mM (Leigh and Wyn Jones, 1984; Marschner, 1995). The high set point for $[K^+]_{\text{cyt}}$ is considered necessary for optimal enzyme activity, as many studies have indicated that $K^+$ is necessary for the activation of a number of cytosolic enzymes, including the plasma-membrane $H^+$-ATPase (Evans and Sorger, 1966; Nitsos and Evans, 1969; Suelter, 1970; Briskin and Poole, 1983; Wyn Jones and Pollard, 1983; O’Neill...
and Spanswick, 1984). Other univalent cations, such as Na\(^+\), Rb\(^+\), Cs\(^+\), and NH\(_4\)^+\), have been shown to be less effective enzyme activators as illustrated by Nitsos and Evans (1969) using starch synthetase. Although in the case of protein synthesis, Na\(^+\) has been shown to replace the function of K\(^+\) effectively (Flowers and Dalmond, 1992). K\(^+\) can modify enzyme function by stabilizing the protein structure more efficiently than other cations (Page and Di Cera, 2006). However, K\(^+\) can be replaced by other cations in this role. Other enzymes are inoperative without K\(^+\), such as pyruvate kinase. In this role, K\(^+\) activates the protein by binding six to eight ligands (often carboxyl oxygen atoms), adjusting the protein conformation (Page and Di Cera, 2006).

General maintenance of the photosynthetic apparatus relies heavily on K\(^+\), and K\(^+\) deficiency reduces photosynthetic activity, chlorophyll content, and carbon translocation within leaves (Hartt, 1969; Peoples and Koch, 1979; Longstreth and Nobel, 1980; O’Toole et al., 1980; Pier and Berkowitz, 1987). Plant movements such as closing or opening of stomata, leaf movements, and other plant tropisms are modulated by K\(^+\)-generated turgor pressure (Moran et al., 1990; Antkowiak et al., 1992; Maathuis and Sanders, 1996a; Philippar et al., 1999). The osmotic pressure produced by K\(^+\) is also utilized in cellular extension and leaf expansion (Green and Muir, 1979; de la Guardia and Benlloch, 1980; Mengel and Arneke, 1982; Maathuis and Sanders, 1996a; Elumalai et al., 2002). K\(^+\) is considered highly mobile within plants, cycling between roots and shoots in the xylem and phloem. This is most evident in its cotransport with nitrate to shoots and subsequent translocation to roots with malate when plants are supplied with NO\(_3^–\), as well as its transport along with amino acids in the xylem (Ben Zioni et al., 1971; Jeschke et al., 1985). K\(^+\) circulation is considered an important source of K\(^+\) in roots, particularly with NO\(_3^–\)-grown plants, and phloem delivered K\(^+\) has been suggested as a signal that can alter K\(^+\) influx (Kirkby and Knight, 1977; Armstrong and Kirkby, 1979; Peuke and Jeschke, 1993;
White, 1997). The relatively high permeability of plant cells to K\(^+\) is reflected in its ability to impose short- and long-term modifications upon the plasma-membrane electrical potential that is established and maintained by the H\(^+\)-ATPase. A decrease or increase of \([K^+]_{\text{ext}}\) has been shown to cause permanent hyperpolarization (upon removal of K\(^+\)) or depolarization (upon addition of K\(^+\)) of the plasma-membrane electrical potential (Etherton and Higinbotham, 1960; Pitman et al., 1970; Cheeseman and Hanson, 1979; Beilby and Blatt, 1986; Newman et al., 1987; Kochian et al., 1989; Maathuis and Sanders, 1996a; Hirsch et al., 1998; Rodriguez-Navarro, 2000; Diatloff et al., 2004). In some plant species, such as rice (\textit{Oryza sativa}) or the halophyte \textit{Triglochin maritima}, NH\(^4\)\(^+\) and Na\(^+\), respectively, have been shown to also adjust the plasma-membrane electrical potential (Jefferies, 1973; Wang et al., 1994). However, in most plants the plasma-membrane electrical potential is only transiently modified by either NH\(^4\)\(^+\) or Na\(^+\) (Higinbotham et al., 1964; L’Roy and Hendrix, 1980; Cheeseman, 1982; Cheeseman et al., 1985).

**Primary K\(^+\) uptake from the external environment**

Nutrient transport has been described as utilizing a variety of transport modes traditionally designated as active transport, cotransport, and uniport mediated (Harvey and Slayman, 1994). While still in use, much of this terminology has been updated to include the latest molecular findings. For instance, active transport describes the primary active transporters that couple ATP hydrolysis to solute movement, such as in H\(^+\) and Ca\(^{2+}\) ATPases found in plant cells. Symporters and antiporters share the common feature of cotransporting two molecular species (ions or molecules). Symporters couple the active transport (against the electrochemical gradient) of one species, to the passive transport (down the electrochemical gradient) of another,
with both species moving in the same direction (either inward or outward). Antiporters couple the active transport of one molecular species in one direction, to the passive transport of another in the opposite direction. Because symporters and antiporters use the potential energy established by active transporters (e.g. the pH gradient established by the H⁺-ATPase), they are also described as secondary active transporters. Uniporters mediate the transport of ions or molecules down their electrochemical gradient. Such transport is now more widely described as channel-mediated transport.

The primary acquisition of K⁺ from the external environment has been described by a biphasic pattern (Fig. 1.1), as the sum of two uptake mechanisms differentiated by: their half-saturation concentration; maximal velocity of K⁺ uptake; sensitivity to transport inhibition; and, types of proteins mediating transport (Gierth and Mäser, 2007; Lebaudy et al., 2007; Britto and Kronzucker, in press). Operating at micromolar concentrations of K⁺, the high-affinity transport system (HATS) has been described as a transport mechanism that facilitates the thermodynamically active entry of K⁺, by coupling its flux to the movement of H⁺ and ATP hydrolysis (Cheeseman et al., 1980; Rodríguez-Navarro et al., 1986; Blatt et al., 1987; Newman et al., 1987; Kochian et al., 1989; Maathuis and Sanders, 1994; Briskin and Gawienowski, 1996). Despite some disagreement concerning the stoichiometry and type of coupling between K⁺ and H⁺, it is generally accepted that K⁺ uptake in plants at low [K⁺]_ext is a symport that is coupled, in a 1:1 ratio, to the passive entry of H⁺, as demonstrated in bacterial (Bakker and Harold, 1980) and fungal systems (Boxman et al., 1984, Rodríguez-Navarro et al., 1986; Blatt et al., 1987). The $K_M$ value for K⁺ HATS ranges from 13 to 130 μM, with $V_{max}$ of between 1.8 to nearly 2000 μmol g⁻¹ h⁻¹, depending on the experimental system investigated (Epstein et al., 1963; Kochian and
**Figure 1.1** HATS and LATS of K\(^+\) uptake. Red dashed curve represents K\(^+\) influx mediated by HATS; Green dashed line represents K\(^+\) influx mediated by LATS; Black solid line represents the combined K\(^+\) influx mediated by both HATS and LATS. Adapted from Britto and Kronzucker (in press).
Combined HATS and LATS:
HATS mediated $< 1$ mM $[K^+]_{\text{ext}}$
LATS mediated $> 1$ mM $[K^+]_{\text{ext}}$

LATS
(increasing influx with $[K^+]_{\text{ext}}$)

HATS
($K_M: 13 - 130 \, \mu M; \, V_{\text{max}}: 1.8 - 2000 \, \mu \text{mol g}^{-1} \text{ h}^{-1}$)
Lucas, 1982, 1983; Wrona and Epstein, 1985; Malhotra and Glass, 1995; Maathuis and Sanders, 1996a, b; Santa-María et al., 1997, 2000; Rubio et al., 2000).

HATS-mediated $K^+$ influx has been shown to be inversely correlated with tissue $K^+$ content (Glass 1976, 1978; Glass and Dunlop, 1979; Kochian and Lucas, 1982; Siddiqi and Glass, 1982, 1986; Fernando et al., 1990). Such a mechanism relating ion uptake with tissue content has been observed for a variety of nutrients including $NH_4^+$, $NO_3^-$, $Cl^-$, $SO_4^{2-}$, and $PO_4^{3-}$ (Epstein and Bloom, 2005). HATS-mediated $K^+$ influx is also reduced by $NH_4^+$ (Scherer et al., 1984; Vale et al., 1987, 1988a, b; Wang et al., 1996; Spalding et al., 1999; Santa-María et al., 2000; Bañuelos et al., 2002; Kronzucker et al., 2003b; Martínez-Cordero et al., 2004; Nieves-Cordones et al., 2007). Reduced $K^+$ uptake and accumulation in the presence of $NH_4^+$ is, indeed, considered a hallmark of $NH_4^+$ toxicity (Kirkby and Mengel, 1967; Kirkby, 1968; Van Beusichem et al., 1988; Peuke and Jeschke, 1993; Gerendás et al., 1997; Hirsch et al., 1998; Britto and Kronzucker 2002; Martínez-Cordero et al., 2005). The mechanism by which $NH_4^+$ inhibits $K^+$ influx, however, has not been established and is currently thought to be a result of direct competition for entry between $NH_4^+$ and $K^+$ (Vale et al., 1987; Wang et al., 1996; White, 1996). Similarly, $Na^+$ has been shown to suppress HATS-mediated $K^+$ influx, particularly at millimolar $[Na^+]_{ext}$ (Epstein et al., 1963; Cheeseman, 1982; Jeschke, 1982; Schachtman and Schroeder, 1994; Rubio et al., 1995; Gassmann et al., 1996; Maathuis et al., 1996; Santa-María et al., 1997; Martínez-Cordero et al., 2005; Kronzucker et al., 2006; Nieves-Cordones et al., 2007). However, some studies have suggested that $Na^+$ has little effect (Epstein, 1961; Epstein et al., 1963; Rains and Epstein, 1967b), or indeed may stimulate HATS-mediated $K^+$ influx (Rubio et al., 1995; Spalding et al., 1999). Differences in experimental systems tested (heterologous expression systems, excised roots, or intact plants), along with a multitude of concentration
gradients employed, have led to confusing results regarding the effects of Na⁺ upon HATS-mediated K⁺ influx.

The K⁺ low-affinity transport system (LATS) predominantly functions at high external concentrations (generally above 1 mM, Fig. 1.1), with K⁺ transport considered to be channel-mediated (Epstein et al., 1963; Kochian and Lucas, 1982; Kochian et al., 1985; Gassmann and Schroeder, 1994; Maathuis and Sanders, 1995; White and Lemtiri-Chlieh, 1995; White, 1996; Hirsch et al., 1998). Unlike K⁺ HATS, under these conditions of high [K⁺]_{ext}, uptake of K⁺ is thermodynamically passive (Maathuis and Sanders, 1996a). However, a consequence of the passive uptake of K⁺, which results in a positive charge inside the cell, is the active removal of a H⁺, in order to re-establish electrical neutrality (see Gerendás and Schurr, 1999; Rodríguez-Navarro, 2000); otherwise, K⁺ influx, with channel-mediated rates between 1 x 10⁶ – 1 x 10⁸ ions s⁻¹ protein⁻¹ (Maathuis et al., 1997), would cause a precipitous depolarization and the electrical destruction of the plasma membrane (Britto and Kronzucker, 2006). Therefore, the distinction between K⁺ HATS and LATS solely based upon energy requirement must include the caveat that differentiates between coupling K⁺ and H⁺ influx, to drive K⁺ entry against an electrochemical gradient (HATS), or coupling H⁺ expulsion following K⁺ entry for charge balancing (LATS).

LATS-mediated K⁺ influx can be distinguished from HATS by the lack of down-regulation in K⁺ influx at high external [K⁺]. K⁺ influx mediated by LATS has been shown to increase with higher [K⁺]_{ext}, despite increasing K⁺ in tissue (Kochian and Lucas, 1982; Kochian et al., 1985; Maathuis and Sanders, 1995), and a progressively depolarized plasma-membrane electrical potential (Etherton and Higinbotham, 1960; Pitman et al., 1970; Cheeseman and Hanson, 1979; Beilby and Blatt, 1986; Newman et al., 1987; Kochian et al., 1989; Maathuis and Sanders, 1996a; Hirsch et al., 1998; Diatloff et al., 2004). K⁺ LATS has been described by both
saturable and linear kinetics, with results varying depending upon the experimental approach used; however, “$K_M$” and “$V_{max}$” values are consistently high (Epstein et al., 1963; Kochian and Lucas, 1982, 1983; Kochian et al., 1985; Wrona and Epstein, 1985; Malhotra and Glass, 1995; Fu and Luan, 1998). Early disagreement regarding the transporters mediating $K^+$ LATS led to various interpretations of the uptake kinetics shown in Figure 1.1, with some researchers claiming the linear uptake of $K^+$ was an artifact or a representation of the serial arrangement of the cytosol and vacuole (Torii and Laties, 1966; Osmond and Laties, 1968; Borstlap, 1981, 1983; Dalton, 1984; Nissen, 1987, 1989). Subsequent identification and characterization of the likely molecular mediators of $K^+$ LATS has removed much of the disagreement concerning $K^+$ LATS as a unique uptake mechanism, despite recent discoveries of ion transporters that display dual-affinity kinetics, mediating ion transport across micro- and millimolar external concentrations (Hirsch et al., 1997; Fu and Luan, 1998, Liu et al., 1999).

LATS-mediated $K^+$ influx has been shown to be sensitive to a variety of channel inhibitors, supporting the hypothesis that it is a channel-mediated transport system. Pharmacological agents that have been extensively tested in animal systems (Hille, 1992), including TEA, Cs$^+$, Ba$^{2+}$, Ca$^{2+}$, and La$^{3+}$, along with molecules such as quinidine, have helped demonstrate the common existence of $K^+$ channels in both plant and animal kingdoms (Leonard et al., 1975; Tester, 1988a, b; Ketchum and Poole, 1990; Blatt, 1992; Wegner et al., 1994; Roberts and Tester, 1995; White and Lemtiri-Chlieh, 1995; Nocito et al., 2002).

Unlike HATS-mediated $K^+$ influx, LATS does not share the characteristic of NH$_4^+$ sensitivity (Spalding et al., 1999; Santa-Maria et al., 2000; Kronzucker et al., 2003b). Nutritional studies have shown that increasing $[K^+]_{ext}$ (into concentrations that are dominantly LATS-mediated) can alleviate the symptoms of NH$_4^+$ toxicity that appear at lower $[K^+]_{ext}$ (Barker et al.,
1967; Ajayi et al., 1970; Dibb and Welch, 1976; Mengel et al., 1976; Lips et al., 1990; Feng and Barker, 1992; Cao et al., 1993; Gerendás et al., 1995; Spalding et al., 1999; Santa-María et al., 2000; Kronzucker et al., 2003b). It has been suggested that $K^+$ may alleviate $NH_4^+$ toxicity by outcompeting $NH_4^+$ at the transport level, as the two ions may share a common transporter, since both are univalent cations with similar hydrated atomic radii (Kielland, 1937; Wang et al., 1996; White, 1996; Nielsen and Schjoerring, 1998; Hess et al., 2006).

Similar to HATS-mediated influx, $Na^+$ has been shown to suppress $K^+$ influx mediated by LATS (Rains and Epstein, 1967b; Benlloch et al., 1994; Flowers and Hajibagheri, 2001; Fuchs et al., 2005; Kronzucker et al., 2006; Wang et al., 2007). It remains unclear why $K^+$ LATS is $Na^+$-sensitive. $Na^+$ may directly inhibit $K^+$ uptake, perhaps because $Na^+$ itself is utilizing $K^+$ LATS transporters (Wang et al., 2007), or $K^+$ flux inhibition may be a result of decreased expression of transporters that mediate $K^+$ LATS influx (Golldack et al., 2003).

**Molecular identities of membrane transporters**

A number of proteins have been identified that may participate in HATS-mediated $K^+$ influx. Grouped into three families (HAK/KUP/KT ($K^+$, $H^+$ symporters), HKT/TRK ($K^+$, $H^+$ or $K^+$, $Na^+$ symporters), and CPA (cation, $H^+$ antiporters)), these transporters mediate $K^+$ influx against its concentration gradient (Maathuis and Sanders, 1999; Rodríguez-Navarro, 2000; Mäser et al., 2001; Gierth and Mäser, 2007; Grabov, 2007). However, the precise coupling mechanism that energizes the thermodynamically active influx of $K^+$ with $H^+$ remains unclear.

Members of the HAK/KUP/KT transport family (Fig. 1.2) have been found to mediate the majority of the HATS-mediated influx (Gierth and Mäser, 2007). Initially identified in
**Figure 1.2** Primary $K^+$ transport families. Different $K^+$ transporters are shown using a variety of colours, with related transporters shown using shades of the same colour ($K^+$ channels of the Shaker family are all shades of purple). Also shown for reference are the different types of $H^+$-ATPases. Adapted from Maathuis and Amtmann (1999).
*Escherichia coli* (KUP) as a transporter that was significantly different from previously identified bacterial TRK $K^+$ transporters (Schleyer and Bakker, 1993), homologous amino acid sequences were subsequently identified in the yeast *Schwanniomyces occidentalis* (Bañuelos et al., 1995) and in barley (*Hordeum vulgare*, Santa-María et al., 1997). Functional analysis of HAK1 in *S. occidentalis* determined that it mediated $K^+$ uptake via a symport mechanism with $H^+$ ($K^+, H^+$ symporter, Haro et al., 1999), confirming electrophysiological studies that demonstrated $K^+$ uptake at low [$K^+$]$_{ext}$ was coupled to $H^+$ influx in fungal and plant systems (Boxman et al., 1984, Rodríguez-Navarro et al., 1986; Blatt et al., 1987). Supporting the hypothesis that HAK/KUP/KT functions in the acquisition of $K^+$ at low [$K^+$]$_{ext}$, $K^+$ starvation was found to promote *HAK* transcript induction in barley, rice, *A. thaliana*, *Capsicum annum*, *Mesembryanthemum crystallinum*, *Solanum lycopersicum*, and *Phragmites australis* (Santa-María et al., 1997; Bañuelos et al., 2002; Su et al., 2002; Ahn et al., 2004; Armengaud et al., 2004; Martinez-Cordero et al., 2004; Shin and Schachtman, 2004; Gierth et al., 2005; Nieves-Cordones, 2007; Takahashi et al., 2007). Conversely, *HAK* transcript abundance decreased with $K^+$-replete conditions. These findings corroborate tracer flux studies that showed reduced HATS-mediated $K^+$ influx with $K^+$-replete conditions (Glass 1976, 1978; Glass and Dunlop, 1979; Kochian and Lucas, 1982; Siddiqi and Glass, 1982, 1986; Fernando et al., 1990; Malhotra and Glass, 1995). $K^+$ influx mediated by HAK/KUP/KT transporters was also found to be reduced by millimolar concentrations of $Na^+$ (Santa-María et al., 1997; Quintero and Blatt, 1997; Fu and Luan, 1998; Su et al., 2002; Martínez-Cordero et al., 2005; Nieves-Cordones et al., 2007). Similarly, $K^+$ uptake studies determined $NH_4^+$ as an inhibitor of $K^+$ influx mediated by HAK/KUP/KT transporters (Santa-María et al., 2000; Bañuelos et al., 2002; Martínez-Cordero et al., 2004, 2005; Vallejo et al., 2005; Nieves-Cordones et al., 2007). Interestingly, a transporter
characterized from *A. thaliana*, AtKUP1, demonstrated the capacity to mediate K$^+$ uptake at both low and high [K$^+$]$_{\text{ext}}$ (Fu and Luan, 1998; Kim et al., 1998). AtKUP1 shares properties of both HAK/KUP/KT and plant K$^+$ Shaker channels (described below). Commonalities include twelve transmembrane-spanning domains (as is characteristic of HAK/KUP/KT transporters) and an amino acid sequence of IYGD (isoleucine-tyrosine-glycine-aspartate), similar to the GYGD/E (glycine-tyrosine-glycine-aspartate/glutamate) found in the pore domain of K$^+$ channels (Chérel, 2004). AtKUP1 shows sensitivity to Na$^+$ and the channel inhibitors TEA, Cs$^+$, and Ba$^{2+}$ (Fu and Luan, 1998). Transporters such as AtKUP1 confirm earlier hypotheses (Nissen 1987, 1989) that a single protein is able to mediate K$^+$ uptake at low and high external concentrations.

Unlike HAK/KUP/KT, the role of the HKT/TRK family (Fig. 1.2) in mediating high-affinity K$^+$ transport in plants has met much debate. Identified as the original plant high-affinity K$^+$ transporter (HKT), it was isolated from a cDNA library derived from K$^+$-deprived wheat (*Triticum aestivum*), sharing sequence similarity with other TRK K$^+$ transporters, and it functionally complemented yeast deficient in K$^+$ uptake (Schachtman and Schroeder, 1994). Measurements of current conductance in *Xenopus* oocytes expressing HKT showed substantial K$^+$ conductance that was superior to NH$_4^+$ or Na$^+$ (Schachtman and Schroeder, 1994). *HKT* mRNA transcripts were localized to wheat root cortical tissue (Schachtman and Schroeder, 1994) and their abundance was shown to increase with K$^+$-deprivation, while Rb$^+$ uptake studies found a pH dependence for transport, supporting the role of HKT in primary high-affinity K$^+$ uptake (Wang et al., 1998). However, K$^+$ transport via HKT has shown a strong dependence upon the expression system used to test its function, and a reliance upon the presence of Na$^+$, with HKT apparently operating as a K$^+$, Na$^+$ symporter at low [Na$^+$]$_{\text{ext}}$ and exclusively as a Na$^+$ transporter at higher [Na$^+$]$_{\text{ext}}$ in *Xenopus* oocytes and yeast (Rubio et al., 1995; Gassmann et al., 1996;
Tests for the presence of coupled K\(^+\), Na\(^+\) symport in intact plants revealed that micromolar [Na\(^+\)] did not stimulate K\(^+\) uptake, or plant growth (Maathuis et al., 1996; Box and Schachtman, 2000). Some evidence remains that supports a limited role for HKT in K\(^+\) uptake, under K\(^+\)-starved conditions (Uozumi et al., 2000; Horie et al., 2001 Garciadeblás et al., 2003; Haro et al., 2005). However, a great body of evidence favours HKT as a mechanism for Na\(^+\) uptake by plants (Uozumi et al., 2000; Horie et al., 2001; Garciadeblás et al., 2003; Kader et al., 2006). Some evidence also suggests a role for HKT in mediating internal Na\(^+\) allocation, particularly in removing Na\(^+\) from the xylem or circulating Na\(^+\) through the phloem (Fairbairn et al., 2000; Mäser et al., 2002a; Berthomieu et al., 2003; Garciadeblás et al., 2003; Su et al., 2003; Gong et al., 2004; Rus et al., 2004, 2006; Sunarpi et al., 2005; Kader et al., 2006; Davenport et al., 2007). Despite numerous examinations of this transporter in various heterologous systems, and new evidence using plant systems, no unequivocal identification of the functional role of HKT in planta has emerged (Maathuis et al., 1996; Hayes et al., 2001; Haro et al., 2005). The diversity of function and dramatic differences in transport specificity or gating properties associated with HKT may be a result of: species differences; variations in the transcript used for analysis; or small changes introduced to the amino acid sequence investigated (Uozumi et al., 1995; Hoth and Hedrich, 1999; Liu et al., 2000; Mäser et al., 2002b; Michard et al., 2005).

Plant cation, proton antiporters (CPA, Fig. 1.2) have been suggested as mediators of K\(^+\) uptake, despite functional analysis describing cation antiporters as transporters regulating cellular ion homeostasis by removing stress-inducing ions (Pardo et al., 2006; Apse and Blumwald, 2007). K\(^+\), H\(^+\) antiporter activity was initially suggested following examinations of plasma membrane vesicles isolated from roots or hypocotyls (Hassidim et al., 1990; Cooper et
al., 1991). Subsequent analyses of putative protein products belonging to the CPA family of proteins determined a wide diversity of transporters, mediating the movement of $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$, and $Cd^{2+}$ (Mäser et al., 2001). The most characterized CPA transporters predominantly mediate the exchange of $Na^+$ with $H^+$, either at the tonoplast (NHX1, Apse et al., 1999), or the plasma membrane (SOS1, Shi et al., 2000). However, the NHX1 transporter has been shown to mediate $K^+$ transport in leaf tonoplast vesicles from tomato plants (*Lycopersicon esculentum*, Zhang and Blumwald, 2001). Moreover, Venema and coworkers (2003) characterized yet another NHX gene from tomato plants (LeNHX2) that coded for a tonoplast-localized $K^+$, $H^+$ exchanger. Recently, Cellier and coworkers (2004) demonstrated increased transcript abundance of a gene (*AtCHX17*) that encodes a putative $K^+$, $H^+$ antiporter, in response to $K^+$ starvation and $Na^+$ stress. While the group hypothesized that the antiporter may function in $K^+$ acquisition, it is difficult to imagine how the transporter would function, since $K^+$ uptake and $H^+$ extrusion would be against both of their respective electrochemical gradients. Shin and Schachtman (2004) also observed $[K^+]_{ext}$-regulated transcriptional activity of the *KEA5* gene, another putative $K^+$ antiporter. Short-term (6 h) $K^+$ deprivation induced *KEA5* transcription; however, transcriptional activity subsequently was reduced with continued low $K^+$ conditions. These results suggest a role for $K^+$, $H^+$ antiporters in cellular $K^+$ homeostasis. However, other members of this family strictly function in the efflux of ions (e.g. $Na^+$) from the cytosolic compartment of plant cells, and future research may find a similar role for these transporters in mediating $K^+$ efflux.

The low-affinity $K^+$ transport system is proposed to be mediated by ion channels (Fig. 1.2). Electrophysiological analyses of guard cells, xylem parenchyma cells, and root protoplasts determined two broad classes of $K^+$-specific channels, either inwardly rectifying channels,
activated by hyperpolarization, or outwardly rectifying channels, activated by membrane depolarization (Lebaudy et al., 2007). Expression studies complementing yeast deficient in K^+ uptake yielded the genetic sequence of the first two inwardly rectifying K^+ channels in plants, *KAT1* and *AKT1* (Anderson et al., 1992; Sentenac et al., 1992). Both *KAT1* and *AKT1*, along with other homologs of these genes, share many genetic similarities with animal Shaker K^+ transporters including: genes encode proteins with six transmembrane domains; a voltage sensor domain is located at the fourth transmembrane domain and contains many basic amino acids; a pore region located between the fifth and sixth transmembrane domains, containing a highly conserved amino acid sequence of GYGD; and a putative cyclic-nucleotide-binding domain located near the C-terminus (Maathuis et al., 1997; Czempinski et al., 1999; Zimmermann and Sentenac, 1999; Chérel, 2004; Gambale and Uozumi, 2006; Gierth and Mäser, 2007; Lebaudy et al., 2007). K^+ Shaker proteins in animal or plant systems have been shown to assemble in the plasma membrane as tetramers (MacKinnon, 1991; Daram et al., 1997). These channels are related to the bacterial KcsA K^+ channel, the first K^+ transporter to be structurally described by x-ray crystallography (Doyle et al., 1998). Unlike high-affinity K^+ transporters, *AKT1* transcript levels do not respond to K^+ starvation in most systems, consistent with its function mediating K^+ uptake at high external [K^+] (Lagarde et al., 1996; Su et al., 2001; Pilot et al., 2003). One notable exception was found by Buschmann et al. (2000), who examined *AKT1* transcript abundance in K^+-starved wheat, suggesting that wheat may not be an ideal system for investigating K^+ transporters (see HKT discussion above). As expected for transporters that mediate K^+ LATS influx, these channels have been found to be inhibited by K^+ channel inhibitors, such as TEA, Ba^{2+}, and La^{3+} (Wegner et al., 1994; Bertl et al., 1995; Müller-Röber et al., 1995; Véry et al., 1995; Lewis and Spalding, 1998; Nielsen and Schjoerring, 1998).
Analysis of the NH$_4^+$ sensitivity of members of the plant Shaker family confirmed previous physiological studies demonstrating the insensitivity of these K$^+$ transporters to external NH$_4^+$ (Bertl et al., 1995; Müller-Röber et al., 1995; White, 1996; Hirsch et al., 1998; Moroni et al., 1998; Spalding et al., 1999; Su et al., 2005). NH$_4^+$-insensitivity by low-affinity K$^+$ transporters was exploited to demonstrate the ability of AKT1 to mediate some high-affinity K$^+$ transport. By inhibiting K$^+$ HATS with NH$_4^+$ in *A. thaliana*, *akt1* mutants were unable to grow at low [K$^+$]$_{ext}$, while wild-type seedlings were unaffected by the low K$^+$ conditions (Hirsch et al., 1998; Spalding et al., 1999). However, it is unclear how other Shaker family members (e.g. SKOR and GORK, Fig. 1.2) respond to NH$_4^+$.

Less definitive has been the role of K$^+$ channels in mediating Na$^+$ fluxes, or the effect of Na$^+$ stress upon K$^+$ channel activity. It has been demonstrated that increasing extracellular Na$^+$ can reduce K$^+$ channel transcript abundance in rice, *A. thaliana* and *M. crystallinum* (Su et al., 2001; Golldack et al., 2003; Pilot et al., 2003). Moreover, it has been suggested that AKT1 functions as a transporter mediating Na$^+$ fluxes (Golldack et al., 2003, Obata et al., 2007; Wang et al., 2007). Interestingly, Qi and Spalding (2004) found that 10 mM [Na$^+$]$_{cyt}$ inhibited AKT1-mediated currents in protoplasts examined using whole-cell patch-clamping, whereas Essah et al. (2003) found no difference in Na$^+$ accumulation in *A. thaliana akt1* mutants versus wild-type seedlings, and Obata et al. (2007) found either no difference in, or lower, Na$^+$ content in yeast and rice cells expressing OsAKT1 as opposed to untransformed cells.

A number of regulatory mechanisms have been identified for K$^+$ Shaker channels. Schroeder and Feng (1991) and Su et al. (2005) determined lower current conductance with decreasing [K$^+$]. It was claimed that K$^+$ channels, particularly in guard cells, were inactivated at micromolar [K$^+$], despite evidence demonstrating Shaker channel-mediate currents at low [K$^+$]$_{ext}$.
(Hirsch et al., 1998; Brüggemann et al., 1999). The low $[K^+]_{\text{ext}}$ was suggested to cause a conformational change in the channel’s pore, essentially reducing the pore’s width and ion conductance (Zhou et al., 2001; Hertel et al., 2005; Su et al., 2005). However, the pore size alone does not determine channel activity, only the likeliness of permeability by an ion. Other channel properties, such as ion binding affinity and the modulation of the activation sensor, are key determinants of ion transport (Zhou and MacKinnon, 2004; Lockless et al., 2007).

$\text{Ca}^{2+}$ signalling and protein phosphorylation are providing additional mechanisms of channel regulation in plants. The $K^+$ transporter HvCBT1, which was isolated from barley aleurone cells, and shares some sequence similarity to $K^+$ Shaker channels, was found to directly bind calmodulin in the presence of $\text{Ca}^{2+}$ (Schuurink et al., 1998). $\text{Ca}^{2+}$ may also function by interacting with G proteins, another type of regulatory mechanism of plant $K^+$ channels (Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993; Kelly et al., 1995; Wegner and De Boer, 1997; Wang et al., 2001). Recently, a signal transduction pathway has been elucidated that describes a specific regulatory mechanism for AKT1: the ankyrin domain of AKT1 binds a calcineurin B-like calcium sensor interacting protein kinase (CIPK23), that is targeted by calcineurin B-like calcium sensors, that in turn are activated by $\text{Ca}^{2+}$ (Li et al., 2006a; Xu et al., 2006; Lee et al., 2007). Previously, the function of the ankyrin domain of AKT1 was unknown and only postulated to interact with the cytoskeleton, as described for animal systems (Davis et al., 1991; Bennet, 1992; Mills and Mandel, 1994).

Members of the Shaker family have also been found to modify channel characteristics by forming heteromeric complexes, with plant members assembling functional channels using subunits from different plant tissues (e.g. roots and shoots), or from different plant species (e.g. $A.\ thaliana$ and $S.\ tuberosum$) (Dreyer et al., 1997; Baizabal-Aguirre et al., 1999; Pilot et al.,
2001, 2003; Reintanz et al., 2002; Xicluna et al., 2007). Heteromeric channels were found to have different current conductances and novel sensitivities to $\text{H}^+$, $\text{Cs}^+$, and $\text{Ca}^{2+}$, reflecting the combination of transporter subunits (Dreyer et al., 1997; Baizabal-Aguirre et al., 1999; Reintanz et al., 2002; Xicluna et al., 2007). While the only in planta example of this type of regulation has been observed in protoplasts with AtKC1 and AKT1 heteromers (Dreyer et al., 1997), this feature of Shaker channels may provide a mechanism for adaptation to abiotic stress or rapidly changing environmental conditions by the assembly of novel ion transporters.

Channel-mediated transport has been found to play a crucial role in $\text{K}^+$ fluxes through the xylem and phloem vasculature (Fig. 1.2). Root xylem parenchyma cells were initially found to exhibit sensitivity to $\text{K}^+$ channel inhibitors such as TEA and $\text{La}^{3+}$ (Wegner et al., 1994). Subsequent investigations partially attributed $\text{K}^+$ transport into the xylem to the SKOR channel, an efflux transporter localized to stelar parenchyma cells and a member of the Shaker family (Gaymard et al., 1998). *A. thaliana* seedlings deficient in SKOR activity had a 50% reduction in shoot $\text{K}^+$ content, while the root $\text{K}^+$ content was unaffected (Gaymard et al., 1998). Unlike xylem transport, phloem loading and unloading of $\text{K}^+$ has been described as mediated by the Shaker transporter AKT2 (Marten et al., 1999; Lacombe et al., 2000; Deeken et al., 2000). AKT2 has been localized to the phloem using the $\beta$–glucuronidase reporter gene and in situ hybridization. Unlike *AKT1*, $\text{K}^+$ starvation has been found to increase transcript abundance of *SKOR* and *AKT2*; however, abscisic acid reduces *SKOR* mRNA abundance, while increasing *AKT2* mRNA (Marten et al., 1999; Lacombe et al., 2000, Deeken et al., 2000, 2002; Pilot et al., 2001, 2003). Moreover, plants possessing *akt2* mutations were found to have disrupted loading of photosynthates into sieve tubes, while *Xenopus* oocytes expressing *akt2* genes showed impaired sucrose, $\text{H}^+$ symport function (Deeken et al., 2002).
Contrasting the inward rectifying role of KAT1 in guard cells, rapid removal of K⁺ during stomatal closure has been attributed in large part to the GORK channel (Ache et al., 2000). Like SKOR, GORK is an efflux transporter and part of the Shaker family (Ache et al., 2000). Supporting GORK function in guard cell movements are studies that demonstrated impaired water relations in plants with *gork* gene mutations, or disruptions in the protein regulators of the GORK channel (Hosy et al., 2003; Becker et al., 2003).

In addition to the variety of Shaker channels mediating K⁺ transport in plants, other channel types have been shown to function in K⁺ movement (Fig. 1.2), such as the tandem-pore K⁺ (TPK) channels (Czempinski et al., 1999; Zimmermann and Sentenac, 1999; Mäser et al., 2001, 2002a; Ashley et al., 2006; Lebaudy et al., 2007). The TPK family of transporters, found in plants, animals, and fungal systems, have between two and eight transmembrane domains, with either an individual pore or, more frequently, two pores, separated by two transmembrane domains, with each domain containing a GYGD sequence, similar to Shaker channels (Zimmermann and Sentenac, 1999; Mäser et al., 2001, 2002a; Czempinski et al., 1999; Ashley et al., 2006; Lebaudy et al., 2007). Unlike Shaker channels, current evidence does not support functional heteromeric TPK proteins (Voelker et al., 2006). TPK channels have been identified in roots, leaves, and flowers, localizing to the tonoplast or plasma membrane, with regulatory binding sites for Ca²⁺ and phosphorylation (Czempinski et al., 1997, 2002; Moshelion et al., 2002; Latz et al., 2007). Although a number of putative TPK channels have been identified in plants, function *in planta* has only been determined for two members: AtTPK4, a plasma-membrane channel, participating in K⁺ transport in pollen and the pollen tube (Becker et al., 2004); and TPK1, a tonoplast-localizing channel that is Ca²⁺-activated, pH-sensitive, and voltage-insensitive (Gobert et al., 2007). Based on these characteristics, TPK1 has been
suggested to be the VK (vacuolar K\(^+\)) channel that was previously identified by electrophysiology (Ward and Schroeder, 1994; Allen and Sanders, 1996; Allen et al., 1998; Bihler et al., 2005).

Although not a TPK channel, another two-pore channel (TPC1) has been identified possessing a Shaker-type structure, with twelve, rather than six transmembrane-spanning domains, and showing Ca\(^{2+}\) and K\(^+\) transport capabilities (Fig. 1.2, Furuichi et al., 2001; Peiter et al., 2005). Electrophysiological analysis of this vacuolar-localizing channel in protoplasts determined ion conductances identical to previously identified SV (slow vacuolar) channels (Hedrich and Neher, 1987; Ward and Schroeder, 1994; Allen and Sanders, 1995; Peiter et al., 2005). Moreover, *A. thaliana* mutants either overexpressing the *TPC1* gene or possessing a *TPC1* gene knockout were found to have SV channel conductances either enhanced or silenced respectively (Peiter et al., 2005). Despite a possible molecular identity for the SV channel, its role *in planta* is not well understood. SV channels have been shown to mediate K\(^+\) fluxes into and out of the vacuole (Allen and Sanders, 1996; Ivashikina and Hedrich, 2005), as well as mediating Ca\(^{2+}\) fluxes into the cytosol (Ward and Schroeder, 1994; Allen and Sanders, 1995, 1996; Bewell et al., 1999). However, some evidence indicates that SV channel activity would be inhibited by physiologically relevant vacuolar Ca\(^{2+}\) concentrations (Pottosin et al., 1997, 2004).

Cyclic-nucleotide gated channels (CNGCs, Mäser et al., 2001, 2002a; Trewavas et al., 2002; Ashley et al., 2006), like TPK channels, are emerging as a new class of K\(^+\) transporters. CNGCs share structural homology with Shaker channels, having six transmembrane domains, with a pore domain located between the fifth and sixth transmembrane units (Talke et al., 2003). CNGCs and some Shaker channels also share the characteristic of having transport activated modulated by cyclic nucleotides (Véry and Sentenac, 2003). However, unlike Shaker channels,
CNGCs do not have a consistent pore sequence comparable to GYGD (Talke et al., 2003). Of the identified CNGCs, two have been shown to have equal K\(^+\) and Na\(^+\) conductance (AtCNGC1 and AtCNGC4, Balagué et al., 2003; Hua et al., 2003), and a third has been implicated in K\(^+\) uptake (AtCNGC10, Borsics et al., 2007). However, it has been suggested that CNGCs mainly function in mediating Na\(^+\), Ca\(^{2+}\), or nonselective cation transport in plants, a role that may also describe TPC1 (Maathuis and Sanders, 2001; Demidchik et al., 2002b; Demidchik and Maathuis, 2007).

Based upon physiological studies, employing such techniques as electrophysiological and radioisotopic methodologies, and molecular biological analysis, capitalizing on heterologous expression of genes or through generating a variety of mutants, the acquisition, distribution, and regulation of K\(^+\) has been rigorously examined. These studies have expanded the original proposal of Epstein and coworkers (1963), that K\(^+\) acquisition is mediated by two uptake mechanisms, and fit molecular transporters to the roles of K\(^+\) uptake. Current models attribute much of the K\(^+\) HATS activity to the AtHAK5 transporter (or homologs) and LATS activity to the AtAKT1 transporter (or homologs, Gierth and Mäser, 2007). While less is known of the in planta function of the other putative K\(^+\) transporters, it is clear that K\(^+\) transport occupies a large proportion of plant resources. What has not emerged from the molecular examinations of K\(^+\) transport are explanations for the maintenance of plant K\(^+\) homeostasis, both cellular and whole-plant.

**Cytosolic K\(^+\): an important consideration**

The cytosolic compartment serves as a vital junction that unites intracellular proteins and organelles with the external environment. Strict maintenance of the solute composition of the
cytosol is not a necessity; however, uncontrolled accumulation of ions, such as NH$_4^+$, can induce plant toxicity by disrupting homeostatically maintained ions, such as Ca$^{2+}$, leading to impaired protein activity and cellular functions (Schroeder and Hagiwara, 1989; Irving et al., 1992; Grabov and Blatt, 1997, 1999; Pei et al., 1998; Britto et al., 2001; Li et al., 2006a, b). The cytosolic [K$^+$] has generally been described as homeostatically set at approximately 100 mM, for optimal protein function (Leigh and Wyn Jones, 1984), although this value has been met with some debate, with a more accurate range of 30 to 320 mM reflecting the scope of values that have been determined in plants (Table 1.1). Suppression of the K$^+$ cytosolic pool has been consistently observed under plant-stress conditions, such as excess Na$^+$ and NH$_4^+$ in the root environment, or K$^+$ deprivation (Mills et al., 1985; Hajibagheri et al, 1987, 1988; Speer and Kaiser, 1991; Walker et al., 1996; Flowers and Hajibagheri, 2001; Carden et al., 2003; Halperin and Lynch, 2003; Kronzucker et al., 2003b). However, it is unclear how such modifications to the cytosolic [K$^+$] will affect plant K$^+$ homeostasis. This point has been illustrated recently in discussions concerning K$^+$ translocation from the root to the shoot, as it is uncertain whether the cytosolic or apoplastic [K$^+$] provides the driving force necessary to regulate SKOR channel activity (Gaymard et al., 1998; Johansson et al., 2006; Liu et al., 2006). Similarly, the extent to which K$^+$ efflux to the external environment from the cytosol has not been conspicuously considered during evaluations of HATS- and LATS-mediated K$^+$ uptake mechanisms. It remains unclear how K$^+$ efflux responds to changing external conditions and what role, if any, the homeostatically maintained cytosolic [K$^+$] contributes to regulating K$^+$ efflux. This is of particular importance under LATS-mediated transport conditions, where it has been found for a number of ions (Na$^+$, NH$_4^+$, NO$_3^-$, and Cl$^-$), that as their external concentration increases, so does their efflux (Britto and Kronzucker, 2006).
Table 1.1 Estimation of the cytosolic $K^+$ concentration using a variety of analytical techniques. 

$^a$ Cytosolic volume estimated to be 5% of cell volume; $^b$ Cytosolic volume estimated to be 3.5% of cell volume; $^c$ Cytosolic volume estimated to be 8.2% of cell volume. Table based upon Britto and Kronzucker, in press.
<table>
<thead>
<tr>
<th>Method</th>
<th>Plant material</th>
<th>$[\text{K}^+]_{\text{cytosol}}$ (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Efflux analysis</strong></td>
<td>Barley root</td>
<td>74-104$^a$</td>
<td>Pitman and Saddler, 1967</td>
</tr>
<tr>
<td></td>
<td><em>Pisum sativum</em></td>
<td>123-166$^b$</td>
<td>Macklon and Higinbotham, 1970</td>
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<tr>
<td></td>
<td>epicotyl</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><em>Avena coleoptile</em></td>
<td>140-215$^b$</td>
<td>Pierce and Higinbotham, 1970</td>
</tr>
<tr>
<td></td>
<td><em>Allium cepa</em> root</td>
<td>113$^c$-184$^a$</td>
<td>Macklon, 1975</td>
</tr>
<tr>
<td></td>
<td><em>Zea mays</em> root</td>
<td>99-108$^b$</td>
<td>Davis and Higinbotham, 1976</td>
</tr>
<tr>
<td></td>
<td><em>Beta vulgaris</em> root</td>
<td>83$^b$</td>
<td>Szynkier and Kylin, 1976</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>236$^a$</td>
<td>Bange, 1979</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>316-320$^a$</td>
<td>Behl and Jeschke, 1982</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>127-187$^a$</td>
<td>Memon et al., 1985a</td>
</tr>
<tr>
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<td><em>Atriplex nummularia</em></td>
<td>90-136$^a$</td>
<td>Mills et al., 1985</td>
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<td></td>
<td>root</td>
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<tr>
<td></td>
<td><em>Avena sativa</em> root</td>
<td>82-238$^a$</td>
<td>Mills et al., 1985</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>32-136$^a$</td>
<td>Kronzucker et al., 2003b</td>
</tr>
<tr>
<td><strong>K$^+$-selective</strong></td>
<td><em>Acer pseudoplatanus</em></td>
<td>126</td>
<td>Rona et al., 1982</td>
</tr>
<tr>
<td><strong>microelectrode</strong></td>
<td>(suspension cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>A. thaliana</em> root</td>
<td>83</td>
<td>Maathuis and Sanders, 1993</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>45-83</td>
<td>Walker et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>39-63</td>
<td>Carden et al., 2003</td>
</tr>
<tr>
<td><strong>X-ray microanalysis</strong></td>
<td><em>Zea mays</em> root</td>
<td>75-162</td>
<td>Hajibagheri et al., 1987, 1988</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>71-119</td>
<td>Pitman et al., 1981</td>
</tr>
<tr>
<td></td>
<td>Barley root</td>
<td>60-132</td>
<td>Flowers and Hajibagheri, 2001</td>
</tr>
<tr>
<td><strong>Fluorescent dye</strong></td>
<td>Barley protoplasts</td>
<td>65-70</td>
<td>Lindberg, 1995</td>
</tr>
<tr>
<td><strong>Cell fractionation</strong></td>
<td><em>Pisum sativum</em> leaf</td>
<td>53</td>
<td>Speer and Kaiser, 1991</td>
</tr>
<tr>
<td></td>
<td><em>Spinacia oleracea</em></td>
<td>147</td>
<td>Speer and Kaiser, 1991</td>
</tr>
<tr>
<td></td>
<td>leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Longitudinal ion profiling</strong></td>
<td>Barley root</td>
<td>110$^a$</td>
<td>Jeschke and Stelter, 1976</td>
</tr>
</tbody>
</table>
Rationale for thesis

Despite numerous advances in our understanding of K\textsuperscript{+} transport, many fundamental questions remain unaddressed, particularly with respect to low-affinity transport. Since the groundbreaking work of Epstein et al. (1963), Glass (1976) and Kochian and Lucas (1982), there has been limited progress in the functional characterization of HATS- and LATS-mediated K\textsuperscript{+} transport, their regulation at the cellular and whole-plant levels, and their relationship to K\textsuperscript{+} homeostasis. It was therefore the goal of the present work to address this deficiency.

My investigations of K\textsuperscript{+} transport, regulation, and homeostasis began with a study on barley that tested the hypothesis, that the maintenance of K\textsuperscript{+} acquisition and compartmentation, are disrupted by NH\textsubscript{4}\textsuperscript{+} in NH\textsubscript{4}\textsuperscript{+}-sensitive barley, which may lie at the heart of the toxicity response in this species (Kronzucker et al., 2003b). The hypothesis was shown to be correct, as high [NH\textsubscript{4}\textsuperscript{+}]\textsubscript{ext} not only reduced K\textsuperscript{+} influx, but also suppressed [K\textsuperscript{+}]\textsubscript{cyt} when [K\textsuperscript{+}]\textsubscript{ext} was low (HATS-mediated K\textsuperscript{+} conditions), compared to lower NH\textsubscript{4}\textsuperscript{+} conditions or NO\textsubscript{3}\textsuperscript{-} controls. By contrast, at higher [K\textsuperscript{+}]\textsubscript{ext} (LATS-mediated K\textsuperscript{+} conditions), NH\textsubscript{4}\textsuperscript{+} did not reduce K\textsuperscript{+} influx, but [K\textsuperscript{+}]\textsubscript{cyt} became independent of N treatment. We concluded that the disruption of K\textsuperscript{+} transport from root to shoot by NH\textsubscript{4}\textsuperscript{+}, with suppression as high as 90\%, is critical to the development of NH\textsubscript{4}\textsuperscript{+} toxicity under HATS-mediated conditions. This suppression was relieved under LATS-mediated conditions, coincident with recovery from NH\textsubscript{4}\textsuperscript{+} toxicity. These findings provided new insight into [K\textsuperscript{+}]\textsubscript{cyt} regulation and whole-plant K\textsuperscript{+} distribution, and raised intriguing new questions about the unique characteristics of the low-affinity K\textsuperscript{+} transport system.

For instance, while increasing K\textsuperscript{+} provision in the LATS range had been shown to result in ever-increasing K\textsuperscript{+} influx into root cells (Epstein et al., 1963; Glass, 1976; Kochian and Lucas,
1982), very little was known about the maintenance of cytosolic K\(^+\) homeostasis under these conditions (Chapter 3). Secondly, the response of K\(^+\) efflux to increasing [K\(^+\)]\(_{\text{ext}}\) was unknown. Did K\(^+\) efflux increase to a very high proportion of K\(^+\) influx in the LATS range, as had previously been demonstrated for NH\(_4^+\) transport and for other ions (Britto and Kronzucker, 2006, Chapter 2, 3)? Would such an increase entail, as with NH\(_4^+\) transport, an energetic burden for the plant (Kronzucker et al., 2001, Chapter 3)? To what extent would changes in the ratio of K\(^+\) efflux to influx, and in the rate of cytosolic K\(^+\) exchange, affect determinations of unidirectional influx (Chapter 2)? How does K\(^+\) supply in the LATS range ameliorate toxic fluxes and accumulation of NH\(_4^+\) in NH\(_4^+\)-sensitive barley (Chapter 4)? Finally, to what extent do K\(^+\) acquisition pathways confer tolerance to NH\(_4^+\) in rice (Chapter 5)?

Using these fundamental questions as a guideline, my thesis examines K\(^+\) transport in the plant species barley and rice. The goal of my work is to gain insight into (1) the pathways mediating, (2) the driving forces affecting, and (3) the energetic consequences of K\(^+\) transport.
CHAPTER 2:

The face value of ion fluxes: the challenge of determining influx in the low-affinity transport range

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*Dr. Kronzucker contributed substantially to the formulation of ideas for this manuscript, as well as to the writing, and data discussions

*Dr. Britto contributed substantially to the writing and to the data discussions
Abstract

The existence of distinct high- and low-affinity transport systems (HATS and LATS) is well established for major nutrient ions. However, influx mediated by these systems is usually estimated using uniformly simple tracer protocols. Here we compare two $^{42}$K$^+$ radiotracer methods to measure potassium influxes in the HATS and LATS ranges in intact barley ($Hordeum vulgare$ L.) roots: a direct influx (DI) method, and compartmental analysis by tracer efflux (CATE), which is designed to account for tracer efflux from labelled roots and differential tracer accumulation along the plant axis. Methods showed only minor discrepancies for influx values in the HATS range, but large discrepancies in the LATS range, revealing striking distinctions in the cellular exchange properties dominated by the operation of the two transport systems. It is shown that accepted DI protocols are associated with very large errors in the high-conductance LATS range, underestimating influx at least six-fold due to four characteristics of this transport mode: 1) accelerated cellular $^{42}$K$^+$ exchange; 2) a greatly increased ratio of efflux to influx; 3) increased $^{42}$K$^+$ loss during removal of water from roots in pre-weighing centrifugation or blotting protocols; and 4) increased $^{42}$K$^+$ retention at the root-shoot interface, a region of the plant frequently disregarded in DI determinations. Our findings warrant a re-evaluation of a large body of literature reporting influx in the LATS range, and are of fundamental importance to ion flux experimentation in plant physiology.
Introduction

The unidirectional influx of nutrient ions into the plant cell is the membrane-transport parameter of greatest interest to most researchers in the field of plant ion transport. This emphasis on influx is entirely reasonable, as it is the primary step in plant mineral acquisition from the external environment. The elucidation of influx patterns in plant roots has led to the identification and characterization of the enzymatic basis of ion transport in these tissues, including the determination of velocity and affinity constants \( V_{\text{max}} \) and \( K_M \), respectively, for distinct transport systems (Epstein et al., 1963). In the present day, the application of accurate influx data is essential to the assignment of specific transport functions to gene products.

By comparison, information about ionic efflux from plant cells is available only for a limited number of conditions and systems. Under steady-state conditions, the use of protocols that allow an investigator to quantify both influx and efflux, has revealed intriguing aspects of ion exchange in plant cells (Jeschke and Stelter, 1973; Cram and Laties, 1974; Kochian and Lucas, 1982; Lee and Clarkson, 1986; Siddiqi et al., 1991; Lasat et al., 2000; Britto et al., 2001, 2004; Kronzucker et al., 1997, 2003a, b), although only a few molecular mechanisms underlying efflux processes have so far been identified (Gaymard et al., 1998; Shi et al., 2000; Mäser et al., 2001; Zhu, 2001). Information about ionic efflux from plant cells might be viewed as being of peripheral interest to the influx analyst, but, as we shall show here, efflux can at times be so high, and cellular ion turnover so rapid, that standard measurements of influx by tracers are not possible without substantial supplemental experimentation.

In this paper, a subsampling CATE protocol is used, which draws upon quantitative efflux data and comprehensive accounting of tracer retention in plant tissue, to demonstrate how
efflux and other factors can strongly impact DI measurements. By examining $^{42}\text{K}^+$-labelled potassium fluxes in the barley model system, we show that the errors delineated have a much greater impact in the LATS range, as compared to the HATS range, powerfully underscoring the fundamental distinctions between these two modes of transport (Epstein et al., 1963; Kochian and Lucas, 1982; Vale et al., 1987; Kronzucker et al., 2003b).

**Materials and Methods**

*Plant culture*

Seeds of barley (*Hordeum vulgare* L. cv. ‘Klondike’) were germinated on sand for 3 d prior to placement on aerated hydroponic growth medium containing modified ¼-strength Johnson’s solution (pH 6.3-6.5) for an additional 4 d. The solution was modified to provide two concentrations of potassium (as $\text{K}_2\text{SO}_4$): 0.1 mM, which is typical of soil $\text{K}^+$ levels and of the operative range of high-affinity $\text{K}^+$ transport (HATS); and 40 mM, which is representative of the low-affinity $\text{K}^+$ transport (LATS) range, and similar to apparent $K_M$ values reported by others (Epstein et al., 1963). Plants were cultured in walk-in growth chambers equipped with fluorescent lights (Philips Econ-o-watt, F96T12), providing an irradiation of ~200 $\mu$mol photons m$^{-2}$ s$^{-1}$ at plant height, for 16 h d$^{-1}$. Daytime temperature was 20°C, nighttime temperature was 15°C, and relative humidity was approximately 70%.

*Flux experiments*

For all flux experiments, each replicate consisted of a bundle of five intact plants (except for experiments comparing blotting and centrifugation, in which eight plants were bundled).
Bundles were prepared 1 d prior to the experiment (6 d after the start of germination), by means of a plastic collar made from a 0.5-cm length of tubing. This collar held together the plant stems, just above the interface of seed and stem. A wire clasp was constructed to attach to the collar, and hook the plant bundle to the rim of a beaker filled with 30 ml of experimental solution, sufficient to immerse the roots. This procedure was used to minimize physical disturbance to the plant root system during plant transfer from one vessel to another, and to minimize transfer times.

All experimental solutions (pre-labelling, labelling, dip, desorption) were of the same chemical composition as the growth solutions, with the labelling solutions containing the potassium radioisotope $^{42}\text{K}^+$ ($t_{\frac{1}{2}} = 12.36$ h), provided by the McMaster Nuclear Reactor, in Hamilton, Ontario, Canada. Solutions were mixed using a fine stream of air bubbles.

The main feature of SCATE experiments was the monitoring of efflux by periodic subsampling of a 30-ml volume of growth solution in which labelled, intact barley roots were immersed. This was performed as described in detail elsewhere (Britto et al., 2006). In brief, roots of bundled plants were pre-loaded in non-radioactive growth solution for 5 min, followed by labelling in growth solution containing $^{42}\text{K}^+$ for 1 h. The roots were then dipped for 5 s in non-radioactive growth solution (for removal of radioactivity in surface water), and sequentially placed into two 30-ml desorption solutions for 5 and 25-30 min, respectively. 3-ml aliquots were periodically removed from the desorption vessels (and replaced with non-radioactive solution) to sample the tracer released from the labelled roots; samples were taken every 30 s from the first vessel, and every 30 s from the second vessel for the first 5 min, then every minute for the remainder of the experiment.
Radioactivity released by plants over time in the two desorption vessels was quantified by $\gamma$-counting and by use of the following formula:

$$
cpm_{\text{released}} = cpm_n \left( \frac{v_{\text{vessel}}}{v_{\text{sample}}} \right) + \sum_{i=1}^{n-1} cpm_i
$$

(Eq. 1)

This gives the cumulative cpm released after removal of the $n^{th}$ sample from the desorption solution. It accounts for the ratio of sub-sample volume ($v_{\text{sample}}$) to total beaker volume ($v_{\text{vessel}}$), and for the sum of cpm previously removed from the beaker. Released radioactivity was plotted against time, and best-fit exponential equations of the form $A_t = A_0 (1 - e^{-kt})$ were determined using a non-linear, least-squares regression (Microcal Origin software version 6.0; see Fig. 2.2). In this equation, $A_t$ and $A_0$ represent, respectively, cumulative tracer released at time $t$, and maximal cumulative tracer released over the entire time course. These equations were differentiated with respect to time, to determine tracer efflux. The specific activities of the tracer-releasing compartments were determined from the kinetic constants ($k$ values) revealed in the exponential regressions, and used to calculate chemical efflux from tracer efflux (Siddiqi et al., 1991; Kronzucker et al., 2003b). Net flux was determined using tracer accumulated in plant tissue (root, shoot, and root-shoot interface), and in centrifuged liquid from roots. Kinetic constants were used to correct the net flux for additional efflux that would have occurred had the elution continued beyond the end of the experiment (Kronzucker et al., 2003b). Influx was obtained by summing efflux and net flux terms.
Direct influx experiments (DI) consisted of a 5-min pre-load in non-radioactive growth solution, followed by variable labelling periods in growth solution containing $^{42}\text{K}^+$, and then a 5-s dip followed by a 5-min desorption, both in non-radioactive growth solution. Most experiments (e.g. those in Table 2.1 and Fig. 2.4) employed a 5-min labelling, since this is a typical period used by many researchers conducting DI experiments. In experiments comparing centrifugation and blotting, the eight-seedling bundles were separated into two sets of four seedlings following desorption, with each set then subjected to either centrifugation or blotting.

At the end of all flux experiments, roots and shoots were excised from the root-shoot interface (which consisted of a 1-cm segment along the root-shoot axis, where seedlings were bundled together, and which included the basal part of the shoot and the seed coat), prior to weighing. Roots were spun in 16 x 100 mm test tubes in a low-speed centrifuge for 30 s (except in blotting experiments), to remove surface water prior to determining fresh weight. The test tubes contained a compacted, absorbent paper tissue (Kimwipe) that was used to capture surface water and any residual $^{42}\text{K}^+$ from the roots during centrifugation. Blotting was carried out by placing the roots in a folded Kimwipe, which was then pressed for 10 s with a 750-g mass. Gamma counts from roots, shoots, sub-samples of washing medium, root-shoot interface, and Kimwipes from blotting or centrifugation, were detected using a Canberra-Packard, Quantum Cobra Series II, Model 5003 $\gamma$-counter equipped with a NaI crystal/photodiode detector system. All radioactive samples were presented to the counter in 16 x 100 mm test tubes for 1 min, with an energy window of 1200-1800 keV.

DI values were initially calculated by adding together the counts accumulated in roots and shoots. Corrections were made to this base value first by adding tracer retained in the root-shoot boundary, and recovered from centrifugation or blotting of roots. This quantity was then
corrected for the losses via efflux that occurred during both absorption and desorption according to the following formula:

$$\phi_{oc}^* = \left\{ A + \phi_{co}^* \left[ L - \frac{1}{k} \left( e^{-kD} - e^{-k(t_L + t_D)} \right) \right] \right\} / t_L$$

(Eq. 2)

(Britto and Kronzucker 2001a), where $\phi_{oc}^*$ is the corrected tracer influx, $A$ is tracer retained in plant tissue, $t_L$ is labelling time, $t_D$ is desorption time, $\phi_{co}^*$ is maximal tracer efflux, and $k$ is the rate constant for cytosolic turnover of the ion in question. The parameters $\phi_{co}^*$ and $k$ were determined using SCATE.

Statistical analysis

Statistical analyses were conducted using a paired-sample $t$ test with the statistical package SPSS (ver. 12).

Results and Discussion

Table 2.1 shows the results of DI and SCATE experiments conducted at two external $K^+$ concentrations, one in the HATS (0.1 mM), the other in the LATS (40 mM) range. Values found using DI agree well with prior studies that also used DI to measure potassium fluxes in the steady state (i.e., under growth and assay conditions that are identical to one another; Epstein et al., 1963; Kochian and Lucas, 1982; Wrona and Epstein, 1985; Siddiqi and Glass, 1986). However, at both concentrations, the influx value determined using SCATE was significantly
Table 2.1  Comparison of $^{42}$K$^+$ influx methods in roots of intact barley seedlings, measured in high- and low-affinity transport (HATS and LATS) ranges. DI, direct influx measured by $^{42}$K$^+$ uptake during a short (5 min) labelling period. DI data are uncorrected (see Fig. 2.4 for correction components), reflecting standard procedures. SCATE, involving 60-min labelling followed by the monitoring of efflux as a component of influx. Each datum represents the mean ± SEM of 5 – 33 replicates. Different superscripted letters within a row refer to significantly different values ($P < 0.05$).
<table>
<thead>
<tr>
<th>[K$^+$]$_{ext}$ (mM)</th>
<th>Influx (μmol g$^{-1}$ (root FW) h$^{-1}$)</th>
<th>DI</th>
<th>SCATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (HATS)</td>
<td>7.31 ± 0.18$^{a}$</td>
<td></td>
<td>10.43 ± 0.55$^{b}$</td>
</tr>
<tr>
<td>40 (LATS)</td>
<td>10.30 ± 0.22$^{a}$</td>
<td></td>
<td>74.70 ± 13.78$^{b}$</td>
</tr>
</tbody>
</table>
higher \((P < 0.05)\) than that determined using DI. In the HATS condition, this difference was moderate, but in the LATS condition, it was extremely pronounced, the SCATE-derived value being seven-fold higher than the DI-derived value. Given this sizeable discrepancy, and because the vast majority of ion transport data reported in the literature are obtained by DI protocols, it is urgent that the nature of this discrepancy be understood.

Figure 2.1 shows a short-term time course of direct influx measurements conducted in the LATS range. The severe drop in influx estimates observed over time is indicative of tracer efflux occurring simultaneously with influx and during post-labelling desorption of the free space (Cram, 1969; Lee and Ayling, 1993; Britto and Kronzucker, 2001a), an artifact not seen in the HATS range. Similarly, the method-dependent differences between flux estimates can be partially explained by accounting for the efflux of tracer, a phenomenon that is only directly observed and quantified in the tracer capture enabled by SCATE protocols. Tracer lost during DI experiments can be corrected for by the application of flux and turnover parameters according to Eq. 2 in Materials and Methods (also see Britto and Kronzucker, 2001a). The cytosolic turnover rate and the ratio of efflux to influx are the two key physiological parameters that determine the extent of the underestimate of influx occurring in DI experiments, and both must be determined independently using SCATE. Figure 2.2 shows the results of two SCATE experiments, one examining the HATS, and the other the LATS, condition. Equation 2 predicts that, when turnover rates and efflux:influx ratios are large, there will be major discrepancies between uncorrected DI and SCATE data. When the steady-state external \([K^+]\) was increased to 40 mM, turnover rates and flux ratios both become large, relative to those at \([K^+]\) concentrations in the HATS range (Fig. 2.2). These increases, as predicted, led to a much greater discrepancy in influx measurements in the LATS range (exchange half-time = 7 min; efflux:influx ratio = 0.8), relative
**Figure 2.1** Short-term time course of uncorrected direct influx (DI) measurements, conducted in the LATS range of $K^+$ transport in intact barley seedlings. Error bars refer to ± SEM of 3 – 33 replicates (n = 33 for the 5 minute time point, which was highly replicated due to its use elsewhere in this study, and to its use as a standard labelling period in other studies).
Labeling Time (min)

Apparent influx (μmol g⁻¹ (root FW) h⁻¹)
Figure 2.2 Representative plots of tracer loss from roots of intact barley seedlings grown and assayed at 0.1 mM (HATS range, filled squares) and 40 mM (LATS, open squares) $[K^+]_{\text{ext}}$. Tracer fluxes were normalized to the same external $^{42}\text{K}^+$ specific activity. Kinetic exchange constants ($k$ values) for HATS and LATS conditions are shown for each tracer. Inset pie-charts demonstrate the relative contribution of efflux (black) to total influx (white plus black) for the two conditions.
Desorption time (min)

Tracer released (cpm g\(^{-1}\) (root FW) \(\times 10^{5}\))

\[ k = 0.037 \quad \text{LATS} \]

\[ k = 0.095 \quad \text{HATS} \]
to the HATS (exchange half-time = 16 min; efflux:influx ratio = 0.2), range. From Eq. 2, it follows that the DI values given for HATS and LATS conditions in Table 2.1 have been underestimated by 7% and 44%, respectively, on account of efflux alone. In the case of $K^+$, the ratio of efflux to influx in the LATS range increases progressively when external $[K^+]$ increases from the HATS range towards the values shown here (Fig. 2.2), and therefore errors incurred in DI measurement must also increase along this gradient. Similar shortening of exchange half-times and/or increases in efflux:influx ratios have been observed for other ions such as $NO_3^-$, $NH_4^+$, $Cl^-$, and $SO_4^{2-}$ (Kronzucker et al., 1999; Min et al., 1999; Scheurwater et al., 1999; ter Steege et al., 1999; Lopez et al., 2002; Kronzucker et al., 2003a; Britto et al., 2001, 2004), and thus similar difficulties with influx determinations in the LATS range are to be expected as the external concentrations of these ions increase. In the case of sodium, which is of primary current interest because of salinity research, and is therefore studied at high external concentrations representing the LATS range, short half-times and high efflux:influx ratios appear to be characteristic (Cheeseman, 1980; Essah et al., 2003; Davenport et al., 2005; Wang et al., 2006). This necessitates the application of caution with respect to interpreting direct influx measurements for these ions, and calls for the complementary execution of SCATE experiments. Even after applying this correction for simultaneous efflux, however, there remained a large discrepancy between LATS influxes measured by DI and by SCATE in the present study.

It is a common practice in flux analysis for researchers to discard the region of the plant at the root-shoot interface, prior to tracer counting or tissue analysis (Vale et al., 1988a, b; Lazof and Cheeseman, 1988a, b; Siddiqi et al., 1989; Kronzucker et al., 1998, 2000). This practice can be partially justified by concerns about tracer contamination of basal shoot portions, or residual seed material, by a loading solution intended to label the roots alone. However, as shown in
Table 2.2, the proportion of radioactivity found in this region not only varies with external $K^+$ supply, but also depends strongly on analytical procedure. In short-term DI measurements in the LATS range, the tracer accumulated at the root-shoot interface was 1.7 times greater than that which accumulated in the entire remainder of the plant. By contrast, this factor was only 0.05 in the HATS range, and 0.48 and 0.16 in SCATE experiments conducted in the LATS and HATS ranges, respectively. These differences are partly attributable to methodological differences between DI and SCATE in the duration of labelling and desorption of roots, but of more physiological importance is the attribution of the differences between HATS and LATS values to a selective-filtration (i.e., salt-restricting) function of this region (Jacoby, 1979; Johanson and Cheeseman, 1983), which accumulates a greater proportion of ions as the external supply increases. In other words, strong tracer accumulation at the root-shoot interface is more characteristic of whole-plant behavior in the LATS range. Abundant evidence for the often substantial, and highly variable, accumulation of tracer in the basal root and basal shoot regions of the plant can be found in the literature (Jacoby, 1964; Shone et al., 1969; Yeo et al., 1977; Johanson and Cheeseman, 1983; Johanson et al., 1983; Drew and Läuchli, 1985; Lacan and Durand, 1996). The contribution of tracer at the root-shoot interface must be included in the total tracer absorbed by the roots and, therefore, counts retained in this region must be subjected to Eq. 2 in the same way as the remainder of the tissue.

Even after considering the effects of efflux and tracer accumulation at the root-shoot interface, however, the difference between flux estimates determined by DI and SCATE in the LATS range still remained substantial. This observation led to an examination of methods used for the superficial drying of roots following tracer desorption, a process required to obtain accurate fresh weight determination. Such removal of surface water is typically accomplished by
Table 2.2 Accumulation of $^{42}$K$^+$ in root-shoot interface relative to $^{42}$K$^+$ in root and shoot of intact barley seedlings, as measured by two methods in high- and low-affinity transport (HATS and LATS) ranges. Values are expressed as a proportion of accumulated root and shoot counts in each treatment (root + shoot counts = 1). Different superscripted letters within a column refer to significantly different values ($P < 0.05$).
<table>
<thead>
<tr>
<th>[K⁺]_{ext} (mM)</th>
<th>DI</th>
<th>SCATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (HATS)</td>
<td>0.05\textsuperscript{a}</td>
<td>0.16\textsuperscript{a}</td>
</tr>
<tr>
<td>40 (LATS)</td>
<td>1.70\textsuperscript{b}</td>
<td>0.48\textsuperscript{b}</td>
</tr>
</tbody>
</table>
blotting (Jacoby, 1964; Cram and Laties, 1971, Jeschke, 1982; Johansen and Cheeseman, 1983; Drew and Läuchli, 1985; Wrona and Epstein, 1985; Lee and Drew, 1986; Cao et al., 1993; Elphick et al., 2001; Davenport et al., 2005) or low-speed centrifugation (Glass and Perley, 1980; Kochian and Lucas, 1982; Kronzucker et al., 2003a, b) of the root tissue. It is generally assumed that surface water removed by either practice will contain negligible quantities of tracer, given a sufficiently long desorption period prior to the weighing procedure. Surprisingly, however, we found that there was substantial tracer leaving the plant under all conditions as a result of centrifugation and blotting methods, although with no significant differences between these two methods in terms of resulting influx or fresh weight estimates (Table 2.3). It was also found that the amount of tracer lost in this manner, relative to tracer retained in the plant tissue, strongly varied with growth condition and labelling time. As depicted in Figure 2.3, the radioactivity collected in the centrifugation step equaled as much as 200% of the tracer that accumulated in roots and shoots, in the case of short-term (30 s) labelling in the LATS range. Exponential-decay modeling of tracer clearance from extracellular phases (Britto and Kronzucker, 2001a) confirms that a 5 min desorption period will clear the surface-film phase (exchange half-time = 10 s) of >> 99% of its tracer content, and will clear the Donnan free space (exchange half-time = 0.79 min) of ~ 99% of its tracer content. This modeling exercise, combined with the observation that the ratio of counts extracted by blotting or centrifugation, to counts remaining in the roots and shoots, is independent of the desorption period (which varied from 5 min to 30 min; data not shown), led to the conclusion that surface-water-removal techniques can lead to substantial losses of tracer from within the cells of the root. Therefore, a more accurate influx estimate must include any counts collected in this manner, in addition to counts retained within, and effluxed from, plant cells; only procedures that normalize fluxes to measures other than fresh weight (e.g.
Table 2.3 Influx estimates corrected for $^{42}$K$^+$ captured in centrifugation or blotting of labelled roots of intact barley seedlings in high- and low-affinity transport (HATS and LATS) ranges. Each datum is the mean of 7 – 8 replicates (± SEM for fresh weights). The same superscripted letters within a given potassium treatment refer to values that are not significantly different ($P < 0.05$).
<table>
<thead>
<tr>
<th>$[\text{K}^{+}]_{\text{ext}}$ (mM)</th>
<th>Method of processing roots</th>
<th>Influx corrected for processing error ($\mu$mol g$^{-1}$ (root FW) h$^{-1}$)</th>
<th>Percent change after correction</th>
<th>Root fresh weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (HATS)</td>
<td>Centrifugation</td>
<td>9.22 ± 0.31$^a$</td>
<td>2.48</td>
<td>0.42 ± 0.02$^a$</td>
</tr>
<tr>
<td></td>
<td>Blotting</td>
<td>8.72 ± 0.19$^a$</td>
<td>1.21</td>
<td>0.41 ± 0.02$^a$</td>
</tr>
<tr>
<td>40 (LATS)</td>
<td>Centrifugation</td>
<td>12.18 ± 0.70$^a$</td>
<td>24.05</td>
<td>0.53 ± 0.10$^a$</td>
</tr>
<tr>
<td></td>
<td>Blotting</td>
<td>10.30 ± 0.89$^a$</td>
<td>17.88</td>
<td>0.59 ± 0.10$^a$</td>
</tr>
</tbody>
</table>
**Figure 2.3** $^{42}$K$^+$ counts collected from centrifugation of labelled roots from intact barley seedlings, grown and assayed at 0.1 mM (HATS range, filled squares) and 40 mM (LATS range, open squares) $[K^+]_{ext}$, relative to counts retained in root and shoot, and as a function of labelling time. Each datum is the mean ± SEM of 6 – 30 replicates. Error bars in the HATS range are smaller than the symbols.
Labeling Time (min) vs. (Ratio of centrifuged tracer to tracer retained in root and shoot) X 100%
dry weight, surface area) are essentially free of this problem. It is worth noting two additional aspects of the experiment shown in Figure 2.3. First, in the LATS range, the contribution of centrifuged tracer relative to tracer remaining in the roots and shoots declined sharply with labelling time; this represents an increasing development of $^{42}$K+-labelled pools in the plant that are resistant to loss by centrifugation (e.g. in the shoot, and in vacuoles of root cells), and indicates that errors incurred by not including these lost counts are much more severe in short-term DI experiments than in longer-term SCATE experiments. Second, there was very little contribution of centrifuged tracer in the case of HATS-range studies, and the relative contribution did not decline with labelling time. This is attributed to the longer cytosolic exchange half-time, and lower tissue potassium content, of the HATS-range plants. This difference may also be indicative of the activity of mechanosensitive channels (Cosgrove and Hedrich, 1991; Ramahaleo et al., 1996; ter Steege and Stulen, 1997; Demidchik et al., 2002b; Qi et al., 2004) operating in LATS ranges, which could cause higher disturbance-enhanced efflux in the LATS, a phenomenon that has been observed by Hommels et al. (1990).

**Conclusion**

This study shows that there are at least four ways in which major errors can be routinely encountered in direct influx analyses. As shown in Figure 2.4, the extent of these errors depends, for potassium, critically on the plant’s transport mode (Kronzucker et al. 2003b), and similar considerations are believed to apply to the uptake of other ions in the LATS range (see above). In the HATS mode, correction of these errors makes a modest, though significant, change in the resulting flux estimates, increasing the face-value flux as determined by classic DI procedures by
Figure 2.4 Development of corrections to direct influx measurements in LATS and HATS (inset) ranges of K⁺ transport in intact barley seedlings. Corrections are for centrifuged tracer (spin), for contributions from the root-shoot interface (RSI), and efflux occurring during labelling and desorption. SCATE data are shown for comparison. Error bars refer to ± SEM of 5–15 replicates.
Apparent influx (µmol g⁻¹ (root FW) h⁻¹)

**HATS**
- DI (uncorrected)
- DI + spin
- DI + spin + RSI
- DI + spin + RSI + efflux
- SCATE

**LATS**
- DI (uncorrected)
- DI + spin
- DI + spin + RSI
- DI + spin + RSI + efflux
- SCATE
about 16%. This situation is in stark contrast to influx measurements in the LATS range, which increase by an extraordinary 600% at 40 mM, after all corrections are applied to the initial DI estimate. Interestingly, for both HATS and LATS conditions, full correction of DI data nevertheless still results in slightly lower fluxes than with SCATE, although this difference is only significant in the case of the HATS range. It is possible that this residual difference is due to increased relaxation of plants in the SCATE study, which undergo less physical handling than plants in the DI study, over the course of the experiment (Britto et al., 2006).

The physiological basis of the modal distinction discussed here, and the measurement errors associated with this distinction, are, at present, reasonably clear for potassium. The carrier mediated transport proteins that catalyse $K^+$ influx in the HATS mode are limited by substrate binding capacity and require energy to transport $K^+$ across a membrane that is more negatively charged (inside negative) than the Nernst potential, $E_K$. These conditions provide a low membrane conductance that is fundamentally different from the channel-dominated condition found in the LATS mode, in which the plasma membrane is depolarized closer to $E_K$, and high conductances are normal (Cheeseman and Hanson, 1979; Beilby, 1985). For other ions, mechanistic distinctions between flux modes are less clear; for instance, it is feasible that sodium influx has no high-affinity transport mode under normal physiological conditions (i.e. conditions where $K^+$ is present), but may instead be catalysed by channels, either nonselective or predominantly $K^+$-selective (Demidchik et al., 2002b; Tester and Davenport, 2003). In such a case, the errors in determining unidirectional influxes associated with the operation of high-conductance pathways may be found at most, or all, levels of substrate provision (see above). The case of anion permeation is also less clear than that of $K^+$ flux. For anions such as $NO_3^-$ and $Cl^-$, influx is likely to be energy-requiring under all circumstances; however, the proportion of
incoming ions that are subsequently effluxed has also been shown to increase with increasing provision (see above).

In summary, the fundamental differences in the generation of errors between K\(^+\) transport modes are attributable to four distinct characteristics of the LATS range: 1) increased efflux relative to influx; 2) increased cytosolic turnover; 3) increased accumulation of tracer at the root-shoot interface; and 4) increased loss of tracer due to centrifugation or blotting. While this conclusion, therefore, poses no major difficulties to studies, past and present, conducted in the HATS range, it underscores the enormous, and previously unknown, difficulties in measuring fluxes in the LATS range. Moreover, it calls into question virtually all direct influx studies that have been conducted in this range, because, for all ions thus far investigated, the ratio of efflux to influx increases as external ion concentrations increase, often approaching unity (Kronzucker et al., 1999, 2003a, b; Min et al., 1999; Scheurwater et al., 1999; ter Steege et al., 1999; Britto et al., 2001, 2004; Lopez et al., 2002). In addition, for many ions the cytosolic exchange half-time appears to shorten with increasing external concentrations (a notable exception is inorganic N; see Britto and Kronzucker, 2001b), resulting in even more pronounced errors. Given the current interest in the functioning of plant ion transport systems in the context of salinity toxicity and tolerance (Tyerman and Skerrett, 1999; Blumwald et al., 2000; Demidchik et al., 2002b; Tester and Davenport, 2003), and given that studies in this area usually examine LATS-range transport modes, the findings here must be considered by researchers who seek a realistic understanding of the primary acquisition of potentially toxic ions by plants. More fundamentally, the accuracy of concentration-dependent influx isotherms should be re-examined in the light of these findings, particularly when the range of the isotherm is extended to high external concentrations. Isotherms generated by use of direct influx protocols that do not take into account the factors
described here will likely incur ever more serious errors as externally applied concentrations increase.
CHAPTER 3:

Rapid, futile K\(^+\) cycling and pool-size dynamics define low-affinity potassium transport in barley

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*Dr. Kronzucker contributed substantially to the formulation of ideas for this manuscript, as well as to the writing, and data discussions

*Dr. Britto contributed substantially to the writing and to the data discussions
Abstract

Using the short-lived radiotracer $^{42}$K$^+$, we present the first comprehensive subcellular flux analysis of low-affinity K$^+$ transport in plants. We overturn the paradigm of cytosolic K$^+$ pool-size homeostasis, and demonstrate that low-affinity K$^+$ transport is characterized by futile cycling of K$^+$ at the plasma membrane. Using two methods of compartmental analysis in intact seedlings of barley (*Hordeum vulgare* L. cv. Klondike), we present data for steady-state unidirectional influx, efflux, net flux, cytosolic pool size, and exchange kinetics, and show that, with increasing external [K$^+$] ([K$^+$]$_{\text{ext}}$), both influx and efflux increase dramatically, and that the ratio of efflux to influx exceeds 70% at [K$^+$]$_{\text{ext}}$ $\geq$ 20 mM. Increasing [K$^+$]$_{\text{ext}}$, furthermore, leads to a shortening of the half-time for cytosolic K$^+$ exchange, to values two to three times lower than are characteristic of high-affinity transport. Cytosolic K$^+$ concentrations are shown to vary between 40 and 200 mM, depending on [K$^+$]$_{\text{ext}}$, on nitrogen treatment (NO$_3^-$ or NH$_4^+$), and on the dominant mode of transport (high- or low-affinity transport), illustrating the dynamic nature of the cytosolic K$^+$ pool, rather than its homeostatic maintenance. Based on measurements of trans-plasma-membrane electrical potential, estimates of cytosolic K$^+$ pool size, and the magnitude of unidirectional K$^+$ fluxes, we describe efflux as the most energetically-demanding of the cellular K$^+$ fluxes that constitute low-affinity transport.

Introduction

Primary potassium uptake by plants has been described as the sum of activities of two distinct membrane transport systems (Epstein et al., 1963; Kochian and Lucas, 1982; Hirsch et
The high-affinity transport system (HATS) operates primarily at low external concentrations (< 1 mM) of K$^+$, and catalyzes an inward flux, against an electrochemical gradient, by use of a K$^+$/H$^+$ symport mechanism (Véry and Sentenac, 2003). The low-affinity transport system (LATS), by contrast, dominates at higher external concentrations (> 1 mM), mostly via the activity of potassium channels (Maathuis and Sanders, 1997). The distinctive characteristics of these systems are adaptive to highly variable soil K$^+$ concentrations (Ashley et al., 2006), which typically range from 0.1 to 6 mM (Adams, 1971), and are often found at much higher values (Reisenauer, 1966). Thus, plant roots engage HATS and LATS transporters to varying degrees (Kochian and Lucas, 1982; Ashley et al., 2006). It is important to study the physiology of these systems because the differential sensitivities of high- and low-affinity transporters to environmental stressors, such as sodium (Na$^+$) or ammonium (NH$_4^+$), can have profound influences on plant survival in the field. For instance, NH$_4^+$ suppresses high-affinity K$^+$ transport (Scherer et al., 1984; Vale et al., 1987, 1988b; Morgan and Jackson, 1984; Hirsch et al., 1998; Spalding et al., 1999; Santa-Maria et al., 2000; Ashley et al., 2006), while low-affinity K$^+$ transport is relatively NH$_4^+$-insensitive, and contributes to the relief from NH$_4^+$ toxicity at high K$^+$ (Santa-Maria et al., 2000; Britto and Kronzucker, 2002; Kronzucker et al., 2003b). Similarly, variable sensitivities of K$^+$ transporters to soil Na$^+$ are critical factors in salinity tolerance (Epstein et al., 1963; Kochian et al., 1985; Tester and Davenport, 2003; Volkov et al., 2004; Kader and Lindberg, 2005).

Here we demonstrate that unidirectional K$^+$ influx systems are only one aspect of a more comprehensive physiological condition or “transport mode”, in which changes from a high- to a low-affinity mode are linked to major shifts in the plant’s cellular ion relations. Some literature reports indicate that the low-affinity mode may display increased efflux (Pettersson and Kasimir-
Klemedtsson, 1990; Kronzucker et al., 2003b) and cytosolic K$^+$ exchange (Pierce and Higinbotham, 1970; Kochian and Lucas, 1982; Kronzucker et al., 2003b), but these fundamental characteristics of K$^+$ transport and compartmentation have, until now, not been comprehensively investigated. It is better established that the electrical potential across the plasma membrane of plant cells progressively depolarizes in response to increasing external K$^+$ (Etherton and Higinbotham, 1960; Higinbotham et al., 1964; Pitman and Saddler, 1967; Cheeseman and Hanson, 1979; Beilby and Blatt, 1986; Newman et al., 1987; Kochian et al., 1989). This electrical effect is intimately related with the voltage sensitivity of inwardly- and outwardly-rectifying K$^+$ channels (Czempinski et al., 1997; Tyerman and Skerrett, 1999; Zimmermann and Sentenac, 1999), and has consequences for the energetics of K$^+$ transport.

It is furthermore not well understood how transport mode influences cytosolic potassium homeostasis. The cytosolic concentration of K$^+$ has been thought to be maintained stringently near 100 mM, via fluxes from potassium pools in the external medium and the vacuole (Leigh and Wyn Jones, 1984; Beilby and Blatt, 1986; Maathuis and Sanders, 1993; Walker et al., 1996; Leigh, 2001; Ashley et al., 2006). However, we have previously demonstrated (Kronzucker et al., 2003b) that [K$^+$]$_{cyt}$ can be reduced significantly when plant roots are exposed to NH$_4^+$. Similarly, others have shown that Na$^+$ stress can depress [K$^+$]$_{cyt}$ (Jeschke and Stelter, 1976; Harvey et al., 1981; Mills et al., 1985; Hajibagheri et al., 1987, 1988, 1989; Flowers and Hajibagheri, 2001; Carden et al., 2003). In addition, our previous data (Kronzucker et al., 2003b) suggested that [K$^+$]$_{cyt}$ may also, surprisingly, have an inverse relationship with [K$^+$]$_{ext}$ under some conditions, such as when the external K$^+$ concentration is increased from a HATS condition (0.1 mM) to a transitional condition between HATS and LATS (1.5 mM; see Kochian and Lucas, 1982; Kochian et al., 1985). These findings warranted a rigorous investigation of the
question: Are increases in $[K^+]_{ext}$ in the LATS range universally associated with deflections in $[K^+]_{cyt}$, and, if so, what is the nature of these deflections?

In the present study, we use a combination of tracer-flux and thermodynamic analyses to characterize these and other key aspects of the low-affinity $K^+$ transport mode. We present results from two methods of subcellular flux analysis in intact barley seedlings with the short-lived radiotracer $^{42}K^+$, both of which obviate the problems associated with the use of $^{86}Rb$ as a non-isotopic tracer for potassium (see Jeschke, 1970), and with effects of tissue excision (Britto et al., 2006). Steady-state potassium influx, efflux, net flux, cytosolic exchange kinetics, and cytosolic concentrations of this essential nutrient ion are reported, and examined in the context of a thermodynamic analysis that draws upon electrophysiological measurements and provides, for the first time, a quantitative appraisal of the energetic cost of unidirectional $K^+$ efflux from the roots of plants. Our study puts forward strong new evidence for the breakdown of cytosolic $K^+$ homeostasis in response to high external $[K^+]$, and the striking demonstration of rapid and futile cycling of potassium at the plasma membrane of plant cells – two characteristics that are shown to define the LATS condition.

**Materials and Methods**

**Plant culture**

Seeds of barley (*Hordeum vulgare* L. cv. ‘Klondike’) were surface-sterilized for 10 min in 1% sodium hypochlorite and germinated under acid-washed sand for 3 d prior to placement in 4-L vessels containing aerated hydroponic growth medium containing modified 1/4-strength Johnson’s solution, at pH 6-6.5, for an additional 4 d. The solution was modified to provide five
concentrations of potassium (as K$_2$SO$_4$), at 0.1, 1.5, 5, 20, and 40 mM, and either NO$_3^-$ (as Ca(NO$_3$)$_2$) or NH$_4^+$ (as (NH$_4$)$_2$SO$_4$), at 10 mM. Solutions were exchanged frequently (every one to two days) to ensure plants were at a nutritional steady state. Plants were cultured in walk-in growth chambers under fluorescent lights (Philips Econ-o-watt, F96T12), with an irradiation of 200 $\mu$mol photons m$^{-2}$ s$^{-1}$ at plant height, for 16 h d$^{-1}$. Daytime temperature was 20°C, nighttime temperature was 15°C, and relative humidity was approximately 70%.

Flux experiments

An elution method (CATE) and a subsampling method (SCATE) were used to estimate subcellular fluxes and compartmental pool sizes (for details see Appendix I; Lee and Clarkson 1986; Siddiqi et al., 1991; Kronzucker et al., 1995d, 2003b; Britto et al., 2001, 2006). With both methods, each replicate consisted of five plants held together at the shoot base by a plastic collar. Roots of these plants were labelled for 60 min in growth solution containing the radiotracer $^{42}$K$^+$ ($t_{1/2} = 12.36$ h), and then desorbed for 30 min to measure efflux – either by a complete, periodic, exchange of the non-radioactive solution bathing the roots (elution method), or by removing aliquots of the external medium as it becomes progressively labelled (subsampling method; for more details, see below and Britto et al., 2006). Labelling and desorption solutions were identical to growth solutions. $^{42}$K$^+$ was provided by the McMaster University Nuclear Reactor (Hamilton, Ontario, Canada). Solutions were mixed using a fine stream of air bubbles. All radioactive samples were analyzed using a $\gamma$-counter, corrected for isotopic decay during experimentation (Canberra-Packard, Quantum Cobra Series II, Model 5003).

In elution experiments, labelled seedlings were attached to efflux funnels and washed with successive 13-mL aliquots of desorption solution, identical to the growth solution. Elution
protocol was timed as follows: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once), and 2 min (eight times). Immediately following elution, shoots were detached from roots, and roots spin-dried in a low-speed centrifuge for 30 s prior to weighing. $^{42}\text{K}^+$ from eluates, roots, and shoots, and centrifugates were $\gamma$-counted. Linear regression of the function $\log (^{42}\text{K}^+ \text{efflux})$ vs. time (or, as expressed in natural logarithms, $\ln \varphi_{\text{co}(i)} - kt$, where $\varphi_{\text{co}(i)}$ is $^{42}\text{K}^+$ efflux at elution time $t$, $\varphi_{\text{co}(i)}$ is initial $^{42}\text{K}^+$ efflux, and $k$ is the rate constant of the exponential decline in $^{42}\text{K}^+$ efflux; exchange half-times were found from the equation $t_{\frac{1}{2}} = 0.693/k$) was used to resolve the kinetics of the slowest-exchanging phase in these experiments, which represents tracer exchange with the cytosolic compartment (Macklon, 1975; Kochian and Lucas, 1982; Memon et al., 1985a; Kronzucker et al., 2003b). The slope $k$ of the regression line thus represents the kinetic exchange constant for the cytosol, while the intercept represents initial tracer efflux (Lee and Clarkson 1986; Siddiqi et al., 1991; Kronzucker et al., 1995d). Chemical efflux, $\varphi_{\text{co}}$, was determined from this intercept, divided by the specific activity of the cytosol at the end of the 60-min labelling period (see Kronzucker et al., 2003b). Net flux, $\varphi_{\text{net}}$, was found using total-plant $^{42}\text{K}^+$ retention after desorption (for details see Kronzucker et al., 2003b). Influx, $\varphi_{\text{oc}}$, was calculated from the sum of $\varphi_{\text{net}}$ and $\varphi_{\text{co}}$. Cytosolic $[\text{K}^+]$ was determined using the flux-turnover equation, $[\text{K}^+] = \Omega \cdot \varphi_{\text{oc}} / k$, where $\Omega$ is a proportionality constant correcting for the cytosolic volume being approximately 5% of total tissue (Britto and Kronzucker, 2001a; Kronzucker et al., 2003b).

In subsampling experiments, plant bundles were labelled and desorbed in 30 ml of nutrient solution, except for the 0.1 mM $\text{K}^+$ treatment, in which a 100-ml solution was used, to minimize $\text{K}^+$ depletion. After 1 h of labelling, seedlings were immersed for 5 s in non-
radioactive solution to remove superficial tracer, then transferred sequentially to two desorption vessels for 5 and 25 min, respectively. 3-ml aliquots were removed (and replaced with fresh non-radioactive solution every 30 s) from the first desorption vessel, and every 30 s from the second desorption vessel until the 5-min time point, after which samples were taken every minute. Tracer collected over time was quantified as described by Britto et al. (2006). Plant tissues were processed and γ-counted as described above for elution experiments. $^{42}$K$^+$ released over time was resolved using exponential equations of the form $A_t = A_0 (1 - e^{-kt})$, determined by non-linear, least-squares regression (Britto et al., 2006). In this equation, $A_t$ represents cumulative tracer released at time $t$, and $A_0$ represents maximal tracer released over the entire time course. As with the elution method, the kinetic constant ($k$) for cytosolic exchange was found directly from exponential regressions. Efflux was calculated by differentiating the exponential equation above with respect to time ($dA_t/ dt = k \cdot A_0 \cdot e^{-kt} = \varphi_{co}^* \text{ at } t = 0$) and dividing by the specific activity of the cytosol (Britto et al., 2006). Cytosolic half-time and $[K^+]_{cyt}$, $\varphi_{nets}$, and $\varphi_{oc}$ were calculated as stated above for elution experiments.

*Tissue K$^+$ content*

To measure tissue K$^+$ content, roots of barley seedlings were desorbed for 5 min in 10 mM CaSO$_4$ to remove extracellular K$^+$. Roots and shoots were then separated and weighed. Tissue was oven dried for a minimum of 72 h at 80 – 85°C, reweighed, pulverized, and digested with 30% HNO$_3$ for a minimum of 72 h. K$^+$ concentration was determined using a single-channel flame photometer (Digital Flame Analyzer model 2655-00, Cole-Parmer, Anjou, Quebec).
**Electrophysiology**

Membrane potential differences were measured as described elsewhere (Britto et al., 2001). In short, single roots of intact barley seedlings were secured in a clear plastic cuvette filled with nutrient solution and mounted on the stage of an inverted binocular compound light microscope (Alphaphot, Nikon, Japan). Impalements were typically performed at 2-6 cm from the root tip, using a Huxley-style micromanipulator (MX310R, SD instruments, Grants Pass, Oregon), and membrane potentials were measured using a DUO 773 electrometer (World Precision Instruments, Sarasota, Florida). Microelectrodes were made from single-barreled borosilicate glass micropipettes filled with 3 M KCl, and had tip diameters less than 1 μm (confirmed using scanning electron microscopy). Reference electrodes consisted of borosilicate micropipettes filled with 2% (w/v) agar. Impalements were considered successful only when a steady electrical potential difference was maintained for at least 2 min. Activity coefficients ($\gamma$) for the various growth-medium concentrations of K⁺ were estimated using the Debye-Hückel-Onsager equation, adapted for the monovalent cation K⁺:

$$-\log \gamma = \frac{0.5\sqrt{I}}{1 + \sqrt{I}}$$

where $I$ is ionic strength of the medium (Jander and Blasius, 1988). The cytosolic K⁺ activity coefficient used was 0.77 (Macklon, 1975). The Nernst potential ($E_N$) for K⁺ was determined using the following equation:

$$E_N = \frac{RT}{zF} \ln \frac{a_{ext}}{a_{cyt}}$$

where $R$ is the universal gas constant, $T$ was ambient temperature (293 K), $z$ is the ionic charge of the species (+1 for K⁺), $F$ is the Faraday constant, and $a_{ext}$ and $a_{cyt}$ are the external and cytosolic
activities for K\(^+\), respectively. To determine whether transport of K\(^+\) was active or passive, the Ussing-Teorell equation was used:

\[
\frac{\varphi_{oc}}{\varphi_{oc}} = \frac{a_{ext}}{a_{cyt}} e^{-\frac{RTF}{aRT}}
\]

with \(\varphi_{oc}\) and \(\varphi_{oc}\) representing influx and efflux, respectively, from the cytosolic compartment, \(\Delta \Psi\) the measured membrane potential, and \(a\), \(z\), \(F\), \(R\), and \(T\), as described above.

**Statistical analysis**

Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons meeting the assumptions of the Dunnett’s C exam, (not assuming equal variances), with the statistical package SPSS (ver. 12).

**Results**

Figures 3.1 and 3.2 show results of the two methods (elution and subsampling) used to measure K\(^+\) efflux from barley roots that were grown, labelled, and desorbed at five steady-state external K\(^+\) concentrations (0.1 - 40 mM) and two nitrogen (N) sources (10 mM NH\(_4\)\(^+\) or NO\(_3\)-). Figure 3.1 shows the time-dependence of \(^{42}\)K\(^+\) efflux from elution experiments, in the form of standard semilogarithmic plots of changing \(^{42}\)K\(^+\) release rates over time, which were resolved into three exponential phases of efflux (Siddiqi et al., 1991; Kronzucker et al., 1995d). Linear regression of the slowest-exchanging of these phases, that represents tracer release from the cytosol (Memon et al., 1985a; Kronzucker et al., 2003b), revealed two distinct patterns of efflux, one occurring at high and one at low [K\(^+\)]\(_{ext}\); these patterns were seen for both N sources. One aspect of this dual pattern is the tendency for the half-time of cytosolic K\(^+\) exchange, as
**Figure 3.1** Comparison of $^{42}$K$^+$ efflux patterns, as determined by elution, in roots of barley seedlings grown under five K$^+$ concentrations and two N treatments, NH$_4^+$ (A) and NO$_3^-$ (B). Plots have been corrected for differences in root mass and tracer activity (to the arbitrary value of $2 \times 10^5$ cpm $\mu$mol$^{-1}$). Each point is the mean of 6 – 17 replicates (SEM was, on average < 11 % of the mean). Cytosolic exchange half-times are listed in parentheses (SEM < 15 % of the mean).
A

Log ($^{42}$K efflux (cpm released g$^{-1}$ (root FW) min$^{-1}$))

Elution time (min)

$\text{NH}_4^+$

- 0.1 mM (24.7 min)
- 1.5 mM (13.2 min)
- 5 mM (9.3 min)
- 20 mM (8.6 min)
- 40 mM (8.5 min)

B

Log ($^{42}$K efflux (cpm released g$^{-1}$ (root FW) min$^{-1}$))

Elution time (min)

$\text{NO}_3^-$

- 0.1 mM (16.5 min)
- 1.5 mM (11.8 min)
- 5 mM (9.4 min)
- 20 mM (7.7 min)
- 40 mM (8.6 min)
Figure 3.2 Comparison of $^{42}$K$^+$ accrual in growth solution surrounding roots of barley seedlings grown under five K$^+$ concentrations and two N treatments, NH$_4^+$ (A) and NO$_3^-$ (B). Plots have been corrected for differences in root mass and tracer activity (to the arbitrary value of $2 \times 10^5$ cpm $\mu$mol$^{-1}$). Each point is the mean of 3 – 12 replicates (SEM was, on average, < 10 % of the mean). Cytosolic exchange half-times are listed in parentheses (SEM < 15 % of the mean, except for 0.1 K$^+$/NH$_4^+$, where the SEM was 25 % of the mean).
determined from the slopes of the regressed cytosolic lines, to decline with increasing $K^+$ supply, showing two populations of half-times, 16-25 min in the HATS condition (0.1 mM $[K^+]_{ext}$), and shorter ones in the LATS conditions, which cluster between 8-13 min. Secondly, the y-intercepts of the cytosolic lines increase with increasing $[K^+]_{ext}$, indicating a greater degree of efflux as potassium supply goes up. The one exception to this was the slightly greater efflux seen at 0.1 mM $K^+$ relative to 1.5 mM (a transitional concentration at the low end of the LATS range), in nitrate-grown plants.

Similar trends were found in subsampling experiments, as seen in Figure 3.2, which shows $^{42}K^+$ released from plant roots and accruing in the initially non-radioactive external medium (see Britto et al., 2006, for details of this method). This graph is qualitatively different from that in Figure 3.1, as it shows tracer released progressively over time, rather than a rate per se of tracer release. Nevertheless, the greater tendency for $K^+$ to be lost from the plant under higher $[K^+]_{ext}$ is clearly visible here, under both N conditions. The strong difference between cytosolic $K^+$ exchange half-times under HATS and LATS conditions is also indicated in Figure 3.2, with more rapid exchange again prevailing at higher $K^+$ supply.

Figure 3.3 illustrates the dependence of influx and efflux, and the relative independence of the net flux term, on external $[K^+]$, as determined by elution and subsampling methods, respectively. With both methods, net $K^+$ flux was clearly depressed under $NH_4^+$ nutrition at lower $[K^+]_{ext}$, but otherwise $\varphi_{net}$ was fairly uniform. By contrast, efflux, influx and the ratio of the two dramatically increased with greater $K^+$ provision. Efflux:influx ratios exceeded 70% at 20 mM $[K^+]_{ext}$, and reached maximal values of 85% at the highest $[K^+]_{ext}$ of 40 mM.

While fluxes measured by the two methods showed good agreement overall, subsampling yielded higher influx and efflux values at the two highest $K^+$ conditions (20 and 40 mM), when
Figure 3.3 Comparison of K\(^+\) component fluxes, determined by elution (A) and subsampling (B), at five external K\(^+\) concentrations with two N sources. Bars are divided into net flux (black segments) and efflux (clear segments), which together comprise the influx term. Error bars refer to ± SEM of 3 – 17 replicates.
plants were supplied with nitrate. With the subsampling protocol, we also observed an initial, non-exponential burst of tracer efflux, that depended on external \([K^+]\), N source, and physical handling of the plant (see Britto et al., 2006). Figure 3.4 illustrates this dependence, in the form of a change in the ratio of this efflux burst, captured in the first eluate of the subsampling protocol, to the total root retention of tracer at the end of an experimental run. At the higher \([K^+]_{\text{ext}}\) values (and particularly at 40 mM), this tracer burst was greater with nitrate-grown, relative to ammonium-grown plants. This indicates greater membrane-porosity of NO\(_3^–\)-grown plants at high \([K^+]_{\text{ext}}\), which cannot be accounted for in the elution protocol (Britto et al., 2006), and may therefore explain the discrepancy between methods under these specific conditions.

Figure 3.5 depicts the tissue \(K^+\) content of root, shoot, and total plant, for each of the ten treatments. These results agree with the trend shown for \(\varphi_{\text{net}}\) values (Fig. 3.3), in that \(K^+\) accumulation is suppressed in low-\(K^+\), ammonium-grown plants, and \(K^+\) accumulation is relatively insensitive to \([K^+]_{\text{ext}}\). However, calculations based on a 10% daily growth rate of seedlings (Britto et al., 2001), and 16 hours (equivalent to the light period) of sustained uptake at reported net fluxes (Fig. 3.3) reveal that these fluxes are, under all conditions, in excess of those required to achieve the tissue \([K^+]\) levels reported in Figure 3.5. This might, in part, be attributable to net \(K^+\) uptake not being maximally sustained throughout the day, and indeed may assume negative values (Macduff and Dhanoa, 1996), and, in part because \(\varphi_{\text{net}}\) determined by tracer accumulation represents a “quasi-steady influx to the vacuole” which exceeds the \(K^+\) accumulation rate by the amount of efflux from the vacuole (Cram, 1969).

Cytosolic \(K^+\) concentrations, as determined using the two methods of \(^{42}K^+\)-flux analysis, are shown in Figure 3.6 for the ten growth conditions. With both methods, a distinct pattern of decreasing \([K^+]_{\text{cyt}}\) was observed for plants grown with nitrate and with \([K^+]_{\text{ext}}\) between the HATS
Figure 3.4 Ratio of $^{42}\text{K}^+$ captured in the initial eluate during a subsampling protocol, to tracer remaining in roots after elution. Values are corrected for root mass and tracer activity. Filled squares refer to seedlings grown with $\text{NH}_4^+$; clear squares refer to $\text{NO}_3^-$. Each point is the mean ± SEM of 3 – 16 replicates.
Ratio of tracer captured in initial eluate to tracer remaining in roots vs. External K$^+$ concentration (mM).
Figure 3.5 K⁺ tissue content of seedlings grown at five external K⁺ concentrations and two N sources. Root content is represented by clear bars; shoot content by gray bars, and whole plant content by black bars. Error bars refer to ± SEM of 6 – 12 replicates. Root contents are expressed per gram root, shoot contents are expressed per gram shoot, and whole plant contents are expressed per gram total tissue. Different letters above each bar refer to significantly different values within each tissue type (root, shoot, or whole plant) (P < 0.05).
K⁺ content (µmol g⁻¹ (FW))

NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻ | NH₄⁺ | NO₃⁻
---|---|---|---|---|---|---|---|---|---|---|---|---|---
0.1 | b | a | b | a | b | a | b | a | b | a | b | a | b
1.5 | b | a | b | a | b | a | b | a | b | a | b | a | b
5 | b | a | b | a | b | a | b | a | b | a | b | a | b
20 | e | f | e | f | e | f | e | f | e | f | e | f | e
40 | f | e | f | e | f | e | f | e | f | e | f | e | f

External N treatment/External K⁺ concentration (mM)
Figure 3.6 $K^+$ concentration of root cytosolic compartment for barley seedlings grown at five external $K^+$ concentrations and two N sources ($NH_4^+$ or $NO_3^-$), determined by either elution (A) or subsampling (B) protocols. Error bars refer to ± SEM of 6 – 17 replicates (A) and ± SEM of 3 – 6 replicates (B). Different letters above each bar refer to significantly different values ($P < 0.05$).
condition of 0.1 mM and the lower end of the LATS scale (1.5 and 5 mM). This trend, however, was reversed with further increases of $[K^+]_{\text{ext}}$ (to 20 and 40 mM), with the $[K^+]_{\text{cyl}}$ values rising to 200 mM, dramatically above the HATS baseline. With ammonium feeding, suppression of $[K^+]_{\text{cyl}}$ (relative to the nitrate treatment) was observed in the HATS range, and this suppression was maintained in the low end of the LATS range (up to 5 mM $[K^+]_{\text{ext}}$). Between 5 and 40 mM $[K^+]_{\text{ext}}$, however, a pattern of rising $[K^+]_{\text{cyl}}$ was seen in NH$_4^+$-grown plants, in parallel with NO$_3^-$-grown plants.

Table 3.1 shows electrophysiological measurements of $\Delta\Psi$, confirming the progressive depolarization of $\Delta\Psi$ as $[K^+]_{\text{ext}}$ increases, and we found generally lower $\Delta\Psi$ values found in the presence of NH$_4^+$. Table 3.1 combines compartmentation, flux, and electrophysiological data with Nernst and Ussing-Teorell analysis to determine the direction of active K$^+$ flux across the plasma membrane. The analysis shows that, while influx is the active step in the HATS range, efflux is the energy-requiring step under all LATS conditions.

**Discussion**

*Is cytosolic potassium homeostatically controlled in the LATS?*

It has become accepted that the cytosolic K$^+$ pool of plant cells is maintained homeostatically, with high stringency, at approximately 100 mM (Walker et al., 1996, 1998; Leigh, 2001). This is believed to be necessary for the maintenance of critical enzymatic and osmotic functions, even though studies have shown that concentrations far below 100 mM can be sufficient for maximal enzyme activation of K$^+$-dependent enzymes (Nitsos and Evans, 1969; Memon et al., 1985b) and the osmotic functions of K$^+$ may be assumed by compatible solutes or
Table 3.1 Directly measured plasma-membrane electrical potentials ($\Delta\Psi$) and direction of energy requiring flux, as determined by the Nernst and Ussing-Teorell equations. External concentrations of $\text{NH}_4^+$ and $\text{NO}_3^-$ were 10 mM. Membrane potentials are the means ± SD of 7 – 38 replicates.
<table>
<thead>
<tr>
<th>N treatment</th>
<th>$[K^+]_{\text{ext}}$ (mM)</th>
<th>$\Delta\Psi$ (mV)</th>
<th>Energy-requiring flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4^+$</td>
<td>0.1</td>
<td>-118 ± 10</td>
<td>Influx</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>-123 ± 18</td>
<td>Efflux</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-119 ± 9</td>
<td>Efflux</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-59 ± 6</td>
<td>Efflux</td>
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<tr>
<td></td>
<td>40</td>
<td>-51 ± 9</td>
<td>Efflux</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>0.1</td>
<td>-141 ± 11</td>
<td>Influx</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>-125 ± 12</td>
<td>Efflux</td>
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<tr>
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<td>5</td>
<td>-119 ± 13</td>
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<td>20</td>
<td>-86 ± 17</td>
<td>Efflux</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>-86 ± 10</td>
<td>Efflux</td>
</tr>
</tbody>
</table>
other ions (Harvey et al., 1981). The strict maintenance of the cytosolic potassium pool is thought to be achieved by K\(^+\) fluxes from the vacuole and the external medium, although prolonged K\(^+\) starvation or exposure to toxicants such as Na\(^+\) and NH\(_4\)\(^+\) have been shown to suppress [K\(^+\)]\(_{\text{cyt}}\) (Jeschke and Stelter, 1976; Harvey et al., 1981; Mills et al., 1985; Hajibagheri et al., 1987, 1988, 1989; Walker et al., 1996, 1998; Flowers and Hajibagheri, 2001; Carden et al., 2003; Kronzucker et al., 2003b). Furthermore, closer examination of published studies shows a range of [K\(^+\)]\(_{\text{cyt}}\) values, in the absence of toxicants, between 43 and 320 mM (Pierce and Higinbotham, 1970; Leigh and Wyn Jones, 1984; Memon et al., 1985a). In addition, in prior work, we demonstrated the very surprising finding that raising the external K\(^+\) supply from two HATS conditions of 0.02 and 0.1 mM (under which [K\(^+\)]\(_{\text{cyt}}\) constancy was observed), to a HATS-LATS transitional condition of 1.5 mM (Kochian and Lucas, 1982; Kochian et al., 1985), also brought about a lowering of [K\(^+\)]\(_{\text{cyt}}\) in root cells of barley (Kronzucker et al., 2003b). In the present study, we have undertaken a comprehensive analysis of fluxes and subcellular compartmentation of K\(^+\) in the LATS range of transport, largely to investigate whether this dynamic condition is a general characteristic of the LATS.

Figure 3.6, in agreement with our previous work (Kronzucker et al., 2003b), shows that, in nitrate-grown plants, the K\(^+\)-sufficient, HATS-LATS transitional condition of 1.5 mM presents lowered average cytosolic K\(^+\) concentrations, compared to the (also K\(^+\)-sufficient) HATS condition, dropping by more than 50% when examined via subsampling (Fig. 3.6b). [K\(^+\)]\(_{\text{ext}}\)-dependent [K\(^+\)]\(_{\text{cyt}}\) suppression occurred despite the substantial increases in total tissue [K\(^+\)] in both roots and shoots at 1.5 mM (Fig. 3.5), and runs contrary to the paradigm of [K\(^+\)]\(_{\text{cyt}}\) constancy under K\(^+\)-sufficiency. Higher tissue K\(^+\) at 1.5 mM [K\(^+\)]\(_{\text{ext}}\) indicates that the vacuolar K\(^+\) pool increased even as [K\(^+\)]\(_{\text{cyt}}\) declined. K\(^+\) supply to the cytosol, either from the vacuole or
the external medium, therefore, should not be limiting under these conditions. In this part of the applied range of $[K^+]_{ext}$ (0.1 – 1.5 mM), however, $[K^+]_{ext}$-dependent $[K^+]_{cyt}$ suppression was not observed with $NH_4^+$ as a nitrogen source, but this was because $[K^+]_{cyt}$ was already suppressed in the HATS condition by $NH_4^+$, an ion that has well-known inhibitory effects upon high-affinity $K^+$ transport (Vale et al., 1987, 1988b; Hirsch et al., 1998; Spalding et al., 1999; Santa-María et al., 2000). Interestingly, even though $[K^+]_{cyt}$ remained low in plants grown at 1.5 mM (and 5 mM) $[K^+]_{ext}$, with or without $NH_4^+$, no impairment of growth or visual symptoms of toxicity were observed; on the contrary, plants grown at 1.5 and 5 mM $K^+$ had greater fresh weight in both roots and shoots than those grown at 0.1 mM (not shown; see Britto and Kronzucker, 2002). This indicates that the maintenance of a strict $[K^+]_{cyt}$ value is not essential.

While little change in $[K^+]_{cyt}$ occurred between 1.5 and 5 mM $[K^+]_{ext}$, the dynamic nature of $[K^+]_{cyt}$ again became evident as $[K^+]_{ext}$ was increased beyond 5 mM (Fig. 3.6). At these high external concentrations, a recovery of cytosolic $[K^+]$ was observed, with $[K^+]_{cyt}$ values at 20 mM already exceeding those of the HATS condition, and values at 40 mM approximately doubling those at 20 mM. Our results in barley are further supported by experiments conducted in rice ($Oryza sativa$ L., cv. ‘IR-72’), where $[K^+]_{cyt}$ was found to decline from ~160 mM at 0.1 mM $[K^+]_{ext}$, to a minimum of ~40 mM at 1.5 mM $[K^+]_{ext}$, followed by a dramatic rise to ~250 mM at 40 mM $[K^+]_{ext}$ (data not shown).

The variability in LATS-range cytosolic $K^+$ pools demonstrated here stands in sharp contrast to the relative constancy in the HATS range (Kronzucker et al., 2003b), and reveals a distinguishing characteristic between the two transport modes, which must be related to differences in unidirectional and net flux functions, and furthermore has inescapable consequences for the energetics of plasma-membrane transport (see below).
**Futile $K^+$ cycling across the plasma membrane in the LATS**

Analysis of $K^+$ exchange between the external medium and cytosol was an equally important aspect of this study, because of the role that $K^+$ influx and efflux play in the regulation of $[K^+]_{cyt}$. Our previous work had suggested that an increase in $K^+$ supply brings about increases both in the ratio of efflux to influx, and in the degree of cytosolic $K^+$ cycling. Aspects of this cellular behaviour have been observed for other ions such as $Na^+$ (Cheeseman, 1982; Essah et al., 2003; Davenport et al., 2005), $NH_4^+$ (Min et al., 1999; Britto et al., 2001, 2002; Kronzucker et al., 2003a), $NO_3^-$ (Kronzucker et al., 1999; Min et al., 1999; Scheurwater et al., 1999), and $Cl^-$ (Britto et al., 2004), but have, until now, not been systematically demonstrated for potassium. Figures 3.1 and 3.2 show that, regardless of experimental or analytical procedure, plants grown under the HATS condition of 0.1 mM display a much longer half-time of cytosolic $K^+$ exchange relative to the four LATS conditions examined, with plants grown at the intermediate $[K^+]_{ext}$ of 1.5 mM showing a transitional half-time. This pattern was unaffected by N source, and demonstrates that the turnover of the cytosolic $K^+$ pool is accelerated in the LATS condition.

At 5 mM $[K^+]_{ext}$ and above, the acceleration of cytosolic $K^+$ cycling takes on another striking characteristic: the extent of $K^+$ efflux relative to influx (and to net flux) begins to increase, becoming most dramatic at 20 and 40 mM $[K^+]_{ext}$, regardless of N source. At 40 mM, the ratio of efflux to influx exceeds 80%, indicating the increasing intensity of futile $K^+$ cycling at the plasma membrane, reminiscent of the futile cycling previously reported for $NH_4^+$ in several plant systems (Min et al., 1999; Britto et al., 2001, 2002; Kronzucker et al., 2003b). Importantly for the energetics of transport (see below), this change in the partitioning of influx toward efflux and net flux does not come about merely as a redistribution of a constant influx, but occurs in the context of a greatly increased (channel-mediated) influx. In the most dramatic
case, K\(^+\) influx into NH\(_4^+\)-fed plants increases by more than 20-fold over the 0.1 - 40 mM [K\(^+\)]\(_{ext}\) range. This is indicative of a switch between the low end of the [K\(^+\)]\(_{ext}\) range, in which NH\(_4^+\)-sensitive HATS transporters preside, to the LATS section of the range, where NH\(_4^+\)-insensitive K\(^+\) channels and non-selective cation channels are the dominant means of K\(^+\) influx (Hirsch et al., 1998; Santa-Maria et al., 2000; Véry and Sentenac, 2002), and can sustain K\(^+\) acquisition even when external [NH\(_4^+\)] is high, alleviating the toxicity of the NH\(_4^+\) ion (Britto and Kronzucker, 2002). By contrast with influx and efflux trends, there is little increase in the net K\(^+\) flux across the conditions examined, as paralleled by the very moderate increases in tissue K\(^+\) across the same conditions (Fig. 3.5; see Asher and Ozanne, 1966). Thus, the degree of luxury K\(^+\) uptake engaged in by the roots becomes very pronounced. A consequence of very high ratios of efflux to influx is that unidirectional K\(^+\) influx, when measured by protocols that do not quantify efflux, becomes increasingly difficult to measure as [K\(^+\)]\(_{ext}\) rises (Cram, 1969; Britto and Kronzucker, 2001a; see also Essah et al., 2003; Davenport et al., 2005). This problem is exacerbated as K\(^+\) exchange half-times decrease in the LATS range (Britto and Kronzucker, 2001a). Even in our present measurements, that use efflux detection by radiotracing, there are some discrepancies between the two methods used, when [K\(^+\)]\(_{ext}\) becomes very large (20-40 mM), and when NH\(_4^+\) is the nitrogen source.

**Energetics of the LATS flux condition**

Across the ten conditions studied here, the [K\(^+\)]\(_{ext}\)-dependent variations in electrical polarization of the plasma membrane (Table 3.1), with increasing depolarization at higher [K\(^+\)]\(_{ext}\), are in broad agreement with published values (Etherton and Higinbotham, 1960; Higinbotham et al., 1964; Pitman and Saddler, 1967; Cheeseman and Hanson, 1979; Beilby and
Blatt, 1986; Newman et al., 1987; Kochian et al., 1989). This trend, in conjunction with our reported cytosolic K\textsuperscript{+} pool-size dynamics (Fig. 3.6), and highly variable K\textsuperscript{+} fluxes into and out of the cytosol (Fig. 3.3), results in a wide range of conditions governing the energetics of plasma-membrane K\textsuperscript{+} transport. However, as shown in Table 3.1, all experimental treatments except for the HATS condition of 0.1 mM [K\textsuperscript{+}]_{ext} reveal that, of the two dominant fluxes, (unidirectional influx and efflux), efflux is the active, energy-requiring transport step. This scenario is analogous to that prevailing in plants exposed to elevated concentrations of NH\textsubscript{4}\textsuperscript{+} or Na\textsuperscript{+}, where passive, channel-mediated influx (“leak”) is accompanied by active efflux (“pump”) of the ions (Cheeseman 1982; Britto et al., 2001; Kronzucker et al., 2001; Essah et al., 2003; Davenport et al., 2005). In the case of Na\textsuperscript{+}, this extrusion mechanism is thought to occur predominantly via Na\textsuperscript{+}/H\textsuperscript{+} antiporters, known as SOS transporters (Apse and Blumwald, 2002). For potassium, the operation of K\textsuperscript{+}/H\textsuperscript{+} antiporters has also been demonstrated (Hassidim et al., 1990; Cooper et al., 1991), although a molecular identification of these transporters in the plasma membrane is as yet outstanding (Pardo et al., 2006). The identity and contribution of such active transporters to K\textsuperscript{+} efflux are as important to the low-affinity transport condition as are the primary means of K\textsuperscript{+} influx, and therefore need to be investigated with rigor.

The specific energy requirements for K\textsuperscript{+} efflux under each condition in our study are shown in Figure 3.7. While elution and subsampling methods yield different absolute energy values (due to differences in flux and pool-size estimates between methods), the overall pattern is very similar: efflux under all LATS-range conditions requires energy, with the energy demand for K\textsuperscript{+} efflux in nitrate-grown plants substantially exceeding that in ammonium-grown plants when [K\textsuperscript{+}]_{ext} ≥ 20 mM. The more depolarized plasma membrane in NH\textsubscript{4}\textsuperscript{+}-grown plants underlies the lesser energy requirement for LATS-range K\textsuperscript{+} efflux in the presence of NH\textsubscript{4}\textsuperscript{+}. 
Figure 3.7 Energy necessary to drive $K^+$ efflux from barley root cells, as determined by either elution (A) or subsampling (B) protocols. Negative values correspond to passive efflux, while positive values correspond to an active efflux. Filled squares refer to NH$_4^+$ grown plants and clear squares refer to NO$_3^-$ grown plants.
Elution

Subsampling
We previously demonstrated that barley plants suffering from NH$_4^+$ toxicity actively extrude NH$_4^+$ from the cytosolic compartment of root cells, and we partially attributed the toxic effect to this energy demand (Britto et al., 2001; Kronzucker et al., 2001). Calculation shows that, at higher [K$^+$]$_{ext}$ in the presence of NO$_3^-$, the energy requirement for K$^+$ efflux is in fact greater than that for NH$_4^+$. However, plants in this study show no visible symptoms of toxicity or growth impairment; hence, this additional energy burden does not appear to have ill consequences. This is evidently because, unlike plants grown under elevated NH$_4^+$ concentrations and low [K$^+$]$_{ext}$, plants grown with abundant K$^+$ (and with either NH$_4^+$ or NO$_3^-$ as N source) are photosynthetically competent, and indeed display increased rates of photosynthesis and carbon flow to the roots with increases [K$^+$]$_{ext}$ (Hartt, 1970; Viro and Haeder, 1971; Peoples and Koch, 1979; Cakmak, 2005). Thus, the energy-intensive processes of active K$^+$ efflux demonstrated here may have detrimental consequences for plant survival under energy-limiting conditions such as shade growth, given that roots can expend 50-70% of their cellular energy stores towards ion transport (Poorter et al., 1991; Scheurwater et al., 1999). By contrast, under agriculturally common high-irradiance conditions, where there may be an imbalance between the energy absorbed by photosystems and that utilized by metabolism (Hüner et al., 1998), the energy dissipated in futile ion cycling at the plasma membrane may in fact confer a fitness advantage in the field.
CHAPTER 4:

Alleviation of rapid, futile ammonium cycling at the plasma membrane by potassium reveals $K^+$-sensitive and -insensitive components of $NH_4^+$ transport

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*Dr. Kronzucker contributed substantially to the formulation of ideas for this manuscript, as well as to the writing, and data discussions

*Dr. Britto contributed substantially to the writing and to the data discussions

*Dino Balkos assisted with experiments, in addition to collecting and analyzing the data for Table 4.1
Abstract

Futile plasma-membrane cycling of ammonium (NH$_4^+$) is characteristic of low-affinity NH$_4^+$ transport, and has been proposed to be a critical factor in NH$_4^+$ toxicity. Using unidirectional flux analysis with the positron-emitting tracer $^{13}$N in intact seedlings of barley (Hordeum vulgare L.), it is shown that rapid, futile NH$_4^+$ cycling is alleviated by elevated K$^+$ supply, and that low-affinity NH$_4^+$ transport is mediated by a K$^+$-sensitive component, and by a second component that is independent of K$^+$. At low external [K$^+$] (0.1 mM), NH$_4^+$ influx (at an external [NH$_4^+$] of 10 mM) of 92 $\mu$mol g$^{-1}$ h$^{-1}$ was observed, with an efflux:influx ratio of 0.75, indicative of rapid, futile NH$_4^+$ cycling. Elevating K$^+$ supply into the low-affinity K$^+$ transport range (1.5-40 mM) reduced both influx and efflux of NH$_4^+$ by as much as 75%, and substantially reduced the efflux:influx ratio. The reduction of NH$_4^+$ fluxes was achieved rapidly upon exposure to elevated K$^+$, within one minute for influx and within five minutes for efflux. The channel inhibitor La$^{3+}$ decreased high-capacity NH$_4^+$ influx only at low K$^+$ concentrations, suggesting that the K$^+$-sensitive component of NH$_4^+$ influx may be mediated by non-selective cation channels. Using respiratory measurements and current models of ion flux energetics, we discuss the energy cost of concomitant NH$_4^+$ and K$^+$ transport at the root plasma membrane, and its consequences for plant growth. Our study presents the first demonstration of the parallel operation of K$^+$-sensitive and -insensitive NH$_4^+$ flux mechanisms in plants.
Ammonium is present in many terrestrial ecosystems and over a wide concentration range (Pearson and Stewart, 1993; Miller and Cramer, 2005). At low (micromolar) soil concentrations, $\text{NH}_4^+$ is adequate as a sole N source for many plant species (Kronzucker et al., 1997, 1999), but most cannot tolerate millimolar concentrations (Britto and Kronzucker, 2002). In this toxic range, $\text{NH}_4^+$ uptake is mediated by a high-capacity, energetically passive, low-affinity transport system (LATS). However, low-affinity $\text{NH}_4^+$ influx is accompanied by an efflux of $\text{NH}_4^+$ nearly equal in magnitude, resulting in the futile cycling of this ion across the plasma membrane (Britto et al., 2001, 2002; Britto and Kronzucker, 2006). The substantial efflux of $\text{NH}_4^+$ under these conditions has been shown to be energetically costly in $\text{NH}_4^+$-sensitive plant species (Kronzucker et al., 2001), and it has been postulated that a primary cause of $\text{NH}_4^+$ toxicity in plants is the energy lost due to the active removal of $\text{NH}_4^+$ that has entered root cells at an uncontrolled rate (Britto et al., 2001; Kronzucker et al., 2001).

$\text{NH}_4^+$ nutrition has been shown to dramatically influence the mineral composition of plants, particularly in the reduction of cation content (Fig. 3.5; Kirkby and Mengel, 1967; Vale et al., 1987, 1988a; Gerendás et al., 1997; Santa-María et al., 2000). The mechanism underlying this has not been unraveled, but it may be through direct competition between $\text{NH}_4^+$ and other cations for entry through common uptake pathways. In particular, potassium ($K^+$) channels are considered prime candidates for low-affinity $\text{NH}_4^+$ transport, as $\text{NH}_4^+$ and $K^+$ are both monovalent cations with similar hydrated atomic radii (Kielland, 1937; Wang et al., 1996; White, 1996). However, hydrated atomic radius may not be a critical characteristic for use of a
common channel, as the hydrated shell has been shown to be removed as ions pass through the selective filter (Doyle, 2004).

A key relationship between $K^+$ and $NH_4^+$ nutrition is that an increase in external $K^+$ concentration protects sensitive plant species from $NH_4^+$ toxicity (Cao et al., 1993; Spalding et al., 1999; Santa-Maria et al., 2000; Kronzucker et al., 2003b; see Chapter 3). This protection is due in part to the restoration of normal $K^+$ status to the plant, a process that ultimately depends on $K^+$ fluxes into roots and its subsequent translocation to the shoot (Fig. 3.3, 3.5; Kronzucker et al., 2003b). In studies using intact barley seedlings, Kronzucker et al. (2003b) showed that, at low external $K^+$ concentrations, $K^+$ fluxes into the root were much lower in seedlings grown with 10 mM $NH_4^+$ than those grown with 10 mM nitrate ($NO_3^-$), but at high external $K^+$, these fluxes were independent of N source (see also Fig. 3.3). Additionally, the $K^+$ flux from root to shoot, which was the flux most suppressed by $NH_4^+$ at low external $[K^+]$, was nearly identical in plants grown with $NO_3^-$ or $NH_4^+$ at the higher $K^+$ concentration (Kronzucker et al., 2003b). These effects, in particular the suppression of $K^+$ influx at the plasma membrane under low-$K^+$, high-$NH_4^+$ conditions, are likely to be due to the inhibitory action of $NH_4^+$ upon high-affinity KUP/HAK/KT transporters (Spalding et al., 1999).

While inhibition of $K^+$ uptake and modification of $K^+$ efflux by $NH_4^+$ has been demonstrated, the reciprocal effect has been only sparsely investigated (Scherer et al., 1984; Vale et al., 1988b; Wang et al., 1996; Nielsen and Schjoerring, 1998). Nielsen and Schjoerring (1998) found that 100 mM $K^+$ reduced the influx of $NH_4^+$ by 50% in leaf apoplasm of *Brassica napus* L. Other studies have demonstrated moderate suppression of $NH_4^+$ isotherms by $K^+$, but never to the same extent as the suppression of $K^+$ influx by $NH_4^+$ (Scherer et al., 1984; Vale et al., 1988b;
Wang et al., 1996). While many of these studies were conducted at low external concentrations of both NH$_4^+$ and K$^+$, none considered growth conditions mediated by LATS for either ion.

To investigate how NH$_4^+$ fluxes are influenced by external [K$^+$], and how this interaction may underlie potassium’s alleviation of ammonium toxicity, we examined NH$_4^+$ fluxes in intact barley seedlings using the short-lived positron-emitting radiotracer $^{13}$N. We hypothesized that increasing external [K$^+$] would: 1) decrease unidirectional NH$_4^+$ fluxes across the plasma membrane, 2) reduce the high NH$_4^+$ efflux:influx ratio that is symptomatic (and perhaps a causative agent) of NH$_4^+$ toxicity, and 3) lessen the energy burden associated with toxic NH$_4^+$ fluxes. All three hypotheses were borne out in our study. We propose that low-affinity NH$_4^+$ influx is accomplished by two components, the first responding to K$^+$, and the second unaffected by it.

**Material and Methods**

**Plant Culture**

Seeds of barley (*Hordeum vulgare* L. cv. ‘Klondike’) were surface-sterilized for 10 min in 1% sodium hypochlorite and germinated under acid-washed sand for 3 d prior to placement in 4-L vessels containing aerated, ¼-strength Johnson’s solution, at pH 6-6.5, for an additional 4 d. The solution was modified to provide four concentrations of potassium (as K$_2$SO$_4$), at 0.1, 1.5, 5, and 40 mM, and NH$_4^+$ (as (NH$_4$)$_2$SO$_4$), at 10 mM. Solutions were exchanged frequently (every one to two days) to ensure that plants remained at a nutritional steady state. Plants were cultured in a walk-in growth chamber under fluorescent lights (Philips Econ-o-watt, F96T12), with an irradiation of 200 $\mu$mol photons m$^{-2}$ s$^{-1}$ at plant height, for 16 h d$^{-1}$. Daytime temperature was
20°C, nighttime temperature was 15°C, and relative humidity was approximately 70%. On day six (1 d prior to experimentation), seedlings were transferred to an experimental radiotracer facility that had similar irradiance and temperature as the growth chamber.

Compartmental analysis

Compartmental analysis by tracer efflux was used to estimate subcellular fluxes and compartmental pool sizes (for details see Appendix I; Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995d). Each replicate consisted of five plants held together at the shoot base by a plastic collar. Intact roots of these plants were labelled for between 30 and 55 min in solution identical to growth solution but containing the radiotracer $^{15}$N ($t_{1/2} = 9.97$ min; as $^{15}$NH$_4^+$) provided by the CAMH cyclotron facility (University of Toronto, Ontario, Canada). Labelled seedlings were attached to efflux funnels and eluted of radioactivity with successive 20-ml aliquots of nonradioactive desorption solution, identical to the growth solution. The desorption series was timed as follows: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once), and 2 min (eight times). All solutions were mixed using a fine stream of air bubbles. Immediately following elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s prior to weighing. Radioactivity from eluates, roots, shoots, and centrifugates was counted, and corrected for isotopic decay, using a gamma counter (PerkinElmer Wallac 1480 Wizard 3”, Turku, Finland). Linear regression of the function $\ln \varphi_{co(t)}^* = \ln \varphi_{co(i)}^* - kt$ (in which $\varphi_{co(t)}^*$ is tracer efflux at elution time $t$, $\varphi_{co(i)}^*$ is initial radioactive 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; see Fig. 4.1) was used to resolve the kinetics of the slowest-exchanging phase in
these experiments, which represents tracer exchange with the cytosolic compartment (Kronzucker et al., 1995d; Britto and Kronzucker, 2003b). Chemical efflux, \( \varphi_{co} \), was determined from \( \varphi_{co(i)}^* \), divided by the specific activity of the cytosol (\( SA_{cyt} \)) at the end of the labelling period; \( SA_{cyt} \) was estimated by using external specific activity (\( SA_o \)), labelling time \( t \), and the rate constant \( k \), which describes tracer exchange with the cytosol, which are related in the exponential rise function \( SA_{cyt} = SA_o(1 - e^{-kt}) \) (Kronzucker et al., 1995d). Net flux, \( \varphi_{nets} \), was found using total-plant \( ^{15}\text{N} \) retention after desorption (Kronzucker et al., 1995d). Influx, \( \varphi_{oc} \), was calculated from the sum of \( \varphi_{nets} \) and \( \varphi_{co} \). Cytosolic \([\text{NH}_4^+]\) was determined using the flux-turnover equation, \( [\text{NH}_4^+]_{cyt} = \Omega \cdot \varphi_{oc} / k \), where \( \Omega \) is a proportionality constant correcting for the cytosolic volume being approximately 5% of total tissue (Britto and Kronzucker, 2001b).

\( K^+ \) concentration shift experiments followed a protocol identical to the above protocol except that, upon 12.25 min of elution, subsequent aliquots were no longer identical to the growth and labelling solutions, but contained a new \( K^+ \) concentration (0.1 or 5 mM).

**Direct influx**

Influx of \( \text{NH}_4^+ \) was also determined directly, by short-term labelling with \( ^{13}\text{N} \). Seedlings were placed for 5 min in growth solution for equilibration, followed by immersion in labelling solution (containing \( ^{13}\text{NH}_4^+ \)), for either 1 or 5 min (the two labelling times were used to investigate the rapidity of \( \text{NH}_4^+ \)-influx response to changing external \( [K^+] \)). The labelling solution was either identical to the growth solution, for steady-state experiments, or contained a new \( [K^+]_{ext} \), for \( K^+ \) concentration shift experiments. After labelling, plants were transferred to non-radioactive growth solution for 5 s, to reduce tracer carryover to the desorption solution, which was also identical to growth solution, and in which roots were then desorbed for 5 min.
Radioactivity remaining in roots and shoots was quantified by gamma counting. Influx values obtained this way were very close to those determined using compartmental analysis, indicating that the effect of efflux on the measurement of influx was negligible. K$^+$ influx was determined as described for NH$_4^+$, using $^{42}$K$^+$ (provided by the McMaster University Nuclear Reactor, Hamilton, Ontario, Canada), and a single labelling time of 5 min.

**Pharmacological agents**

Similar to the procedure described above, direct influx measurements by short-term labelling with $^{13}$N (or $^{42}$K$^+$) were conducted, in the presence of the channel inhibitors Cs$^+$, La$^{3+}$, and TEA. Seedlings were placed for 10 min in growth solution for equilibration, containing 10 mM Cs$^+$, La$^{3+}$, or TEA. Labelling with $^{13}$N (or $^{42}$K$^+$), and subsequent solution exchanges, were identical to the above procedure except all solutions contained the appropriate channel inhibitor.

**Tissue ammonium determination**

To measure tissue NH$_4^+$ content, barley seedlings were harvested and desorbed for 5 min in 10 mM CaSO$_4$ to remove extracellular NH$_4^+$. Roots and shoots were then separated and weighed, then transferred to polyethylene plastic vials and frozen in liquid N$_2$ for storage at -80°C. Approximately 0.5 g of root or shoot tissue was homogenized under liquid N$_2$ using mortar and pestle, followed by the addition of 6 ml formic acid (10 mM) for the purpose of extracting NH$_4^+$ (Husted et al., 2000). Subsamples (1 ml) of the homogenate were centrifuged at 2.5 x 10$^4$ g (gravitational acceleration) at 2°C for 10 min. The supernatant was transferred to 2 ml polypropylene tubes with 0.45 µm nylon filters (Costar, Corning Inc., USA) and centrifuged at 5 x 10$^3$ g (2°C) for 5 min. The resulting supernatant was analyzed by either the indophenol
colorimetric (Berthelot) method or the o-phthalaldehyde (OPA) method to determine total tissue NH$_4^+$ content. The two analytical methods were used to ensure an accurate range of tissue NH$_4^+$ content was reported, as compounds present in the plant tissue can interfere with the methods, and possibly yield overestimated NH$_4^+$ content values (Husted et al., 2000).

**Indophenol method:** This method has been described in detail elsewhere (Solorzano, 1969; Husted et al., 2000). Briefly, three solutions were combined with 1.6 ml of tissue extract: 1) 200 µl of 11 mM phenol in 95% (v/v) ethanol; 2) 200 µl of 1.7 mM sodium nitroprusside (prepared weekly); and 3) 500 µl of solution containing 100 ml of 0.68 M trisodium citrate in 0.25 M NaOH with 25 ml of commercial strength (11%) sodium hypochlorite. The colour was allowed to develop for 60 min at room temperature (25°C) in the dark, and sample absorbance was measured at 640 nm.

**OPA method:** This method has been described in detail elsewhere for use with spectrophotometry (Goyal et al., 1988). Briefly, 100 ml of OPA reagent was prepared by combining 200 mM potassium phosphate buffer (composed of equimolar amounts of potassium dihydrogen phosphate and potassium monohydrogen phosphate), 3.75 mM OPA, and 2 mM 2-mercaptoethanol one day before use. Prior to the addition of 2-mercaptoethanol, the solution pH was adjusted to 7 with 1 M NaOH, and filtered through a grade 2 Whatman filter paper. 10 µl of tissue extract was combined with 3 ml of OPA reagent, and the colour was allowed to develop in the dark for 30 min at room temperature (25°C), and sample absorbance was measured at 410 nm.
Root respiration and energy cost of transport

Root respiration was determined in intact seedlings using a Hansatech oxygen electrode and Oxygraph control system (Hansatech Instruments, Norfolk, UK). Seedlings were placed in a cuvette with 2.5 ml of air-saturated growth solution. The decline in O$_2$ concentration was monitored for approximately 15 min, but only the initial linear decline was used to calculate O$_2$ depletion rates. The energy costs of ion transport were calculated based upon the following equation:

$$1/U_{\text{theor}} = \frac{[(H/I_j) \times M_j]}{[(H/P) \times (P/O_2)]}$$

Where $1/U_{\text{theor}}$ is the cost of active ion transport (mol O$_2$ mol$^{-1}$ ion), $H/I_j$ is the proton/ion-stoichiometry, $M_j$ is the number of membranes crossed (one in the present instance), $H/P$ is the number of protons pumped by the hydrolysis of one ATP to ADP, and $P/O_2$ is the efficiency of oxidative phosphorylation (Kurimoto et al., 2004). The application of this equation relies on three major assumptions: 1) The transport of any cation or anion is obligatorily coupled to the export of protons by the plasma-membrane ATPase, for charge-balancing purposes, and additionally, in the case of active transport, to maintain the proton gradient that provides energy for the flux. In the present case, LATS-range NH$_4^+$ and K$^+$ transport are assumed to involve an electrogenic uniport that requires charge balancing via the outward pumping of one proton per NH$_4^+$ or K$^+$ entering the cell (see Britto and Kronzucker, 2005, 2006). 2) The stoichiometry of proton export from plant cells via the plasma-membrane H$^+$-ATPase, is 1 ATP hydrolyzed to 1 H$^+$ exported. 3) The phosphorylation ratio that quantitatively links respiratory O$_2$ consumption to ATP production is approximately 5 ATP:O$_2$. Therefore in the application of this model to the primary unidirectional influx of NH$_4^+$ or K$^+$, across the root plasma membrane, $H/I_j = 1$, $H/P = 1$, $P/O_2 = 5$.
and $P/O_2 = 5$, resulting in $1/U_{\text{theor}} = 0.2$ (mol O$_2$ per mol ion transported). This value of $1/U_{\text{theor}}$ was multiplied by the influx of NH$_4^+$ or K$^+$ to determine the theoretical O$_2$ consumed to sustain the flux. For further details on the application of this model to passive cation influx operating concomitantly with active cation efflux and proton pumping, see Britto and Kronzucker (2006).

Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons meeting the assumptions of the Dunnett’s C exam, (not assuming equal variances), with the statistical package SPSS (ver. 12).

Results

Steady state NH$_4^+$ fluxes are strongly affected by K$^+$

Figure 4.1 shows time-dependent efflux of $^{13}$NH$_4^+$ from roots of 7-day-old intact barley seedlings. The semilogarithmic plots displayed a compoundly exponential character, and could be precisely resolved into three kinetically distinct linear phases, each representing tracer released from a separate subcellular compartment (Siddiqi et al., 1991; Kronzucker et al., 1995d; Britto and Kronzucker, 2003b). Slopes of each linear phase yielded half-times of exchange ($t_{1/2}$) for each compartment. The more rapidly exchanging phases, representing the extracellular surface film and Donnan free space, had $t_{1/2}$ values of 7s and 59s, respectively, while the slowest exchanging compartment, identified as the cytosol (Kronzucker et al., 1995d; Britto and Kronzucker, 2003b), had a half-time of 14 min for the high external K$^+$ conditions, and 21 min for the lowest [K$^+$]$_{\text{ext}}$ (Fig. 4.1). Compartment identification was rigorously ascertained in
Figure 4.1 $^{13}$NH$_4^+$ efflux from the roots of intact barley seedlings grown with 10 mM NH$_4^+$ and at four K$^+$ concentrations (as indicated). Each point is the mean of 4 – 9 replicates (SEM was, on average, 15% of the mean). Cytosolic exchange half-times are listed in parentheses (SEM < 8% of the mean).
The graph illustrates the log of $^{15}$NH$_4^+$ efflux (cpm released g$^{-1}$ (root FW) min$^{-1}$) plotted against elution time (min) for different potassium concentrations: 0.1 K$^+$, 1.5 K$^+$, 5 K$^+$, and 40 K$^+$. The data points indicate that the efflux decreases over time, with specific times noted for each concentration: (21.3 min) for 0.1 K$^+$, (14.9 min) for 1.5 K$^+$, and (12.5 min) for 5 K$^+$ and 40 K$^+$. The graph shows a consistent decrease in efflux with increasing potassium concentration.
previous studies (Kronzucker et al., 1995d; Britto and Kronzucker, 2003b), and the magnitude of 
NH$_4^+$ influx as determined using compartmental analysis (Fig. 4.2) was confirmed by direct 
influx measurements (Fig 4.2, inset; refer to Chapter 2).

Root and shoot NH$_4^+$ tissue content was determined using two independent methods, 
indophenol and OPA (Table 4.1). The values obtained by both methods were in excess of what is 
necessary to account for the [NH$_4^+$]$_{cyt}$ estimates calculated by compartmental analysis. Following 
the pattern of changing NH$_4^+$ activity in the cytosol (which dropped from 240-580 mM to 90-150 
mM, depending on activity coefficients used; see Fig. 4.6), raising the external [K$^+$] from 0.1 to 
40 mM dramatically reduced the root tissue content of NH$_4^+$, from 70 to 14 $\mu$mol g$^{-1}$ (root FW).

Net fluxes found with compartmental analysis were similar across treatments, while 
efflux and influx varied dramatically with external K$^+$ supply (Fig. 4.2). At the lowest [K$^+$]$_{ext}$ 
condition of 0.1 mM, under which K$^+$ influx is mediated by a high-affinity transport system, 
NH$_4^+$ influx was significantly greater than at all other conditions, with a rate of 92 $\mu$mol g$^{-1}$ h$^{-1}$.
However, when [K$^+$]$_{ext}$ was elevated into the low-affinity K$^+$ transport range (≥ 1.5 mM), NH$_4^+$ 
influx declined by as much as 63%, to 34 $\mu$mol g$^{-1}$ h$^{-1}$. Even more dramatic was the effect of 
elevated K$^+$ on NH$_4^+$ efflux, which was reduced by as much as 75%, from 69 to 17 $\mu$mol g$^{-1}$ h$^{-1}$.
Because of this differential effect on unidirectional NH$_4^+$ fluxes, the ratio of efflux to influx 
declined substantially when [K$^+$]$_{ext}$ was raised, from 0.75 to as little as 0.42.

Increasing external potassium beyond the LATS threshold value of 1.5 mM did not 
further reduce NH$_4^+$ influx or the efflux:influx ratio. Thus, we identify the constant residual flux 
obscerved throughout the LATS range as the K$^+$-insensitive component of NH$_4^+$ influx.
Table 4.1 Tissue NH$_4^+$ content of roots and shoots of barley seedlings, grown with 10 mM [NH$_4^+$]$_{ext}$, as determined by the indophenol and OPA methods. Each datum represents the mean ± SEM of 6–7 replicates. Different superscripted letters within a column and within a method of NH$_4^+$ determination refer to significantly different values ($P < 0.05$). Data kindly provided by DK Balkos.
<table>
<thead>
<tr>
<th>Assay method</th>
<th>([K^+]_{\text{ext}}) (mM)</th>
<th>Root content ((\mu\text{mol g}^{-1}) (root FW))</th>
<th>Shoot content ((\mu\text{mol g}^{-1}) (root FW))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indophenol (Berthelot)</td>
<td>0.1</td>
<td>69.60 ± 2.66(^a)</td>
<td>43.82 ± 0.72(^a)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>22.60 ± 0.49(^b)</td>
<td>9.47 ± 0.41(^b)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>14.31 ± 0.46(^c)</td>
<td>9.39 ± 0.39(^b)</td>
</tr>
<tr>
<td>OPA</td>
<td>0.1</td>
<td>71.42 ± 1.00(^a)</td>
<td>39.80 ± 1.76(^a)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>22.93 ± 1.46(^b)</td>
<td>10.61 ± 0.19(^b)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>15.07 ± 0.32(^c)</td>
<td>10.72 ± 0.22(^b)</td>
</tr>
</tbody>
</table>
Figure 4.2 Steady-state component fluxes of NH$_4^+$ in roots of barley grown at 10 mM NH$_4^+$ and at four external K$^+$ concentrations (as indicated), as determined by compartmental analysis. Bars are divided into net flux (filled segments) and efflux (clear segments), which together comprise the influx term. Error bars refer to ± SEM of 4 – 5 replicates. Different letters refer to significantly different influx means ($P < 0.05$). Inset. Direct NH$_4^+$ influx measurements using short-term labelling for the four K$^+$ conditions. Error bars refer to ± SEM of 3 – 18 replicates.
NH$_4^+$ flux (µmol g$^{-1}$ (root FW) h$^{-1}$)

External K$^+$ concentration (mM)

- Efflux
- Net flux

Error bars indicate standard deviation.

InInset:

NH$_4^+$ flux (µmol g$^{-1}$ (root FW) h$^{-1}$) vs. External K$^+$ concentration (mM)

- Colour codes:
  - a
  - b

The data shows a significant decrease in NH$_4^+$ flux with increasing external K$^+$ concentration.
**Elevated K⁺ rapidly decreases unidirectional NH₄⁺ influx**

Figure 4.3 shows the influx of NH₄⁺ into intact barley seedlings, as determined by short-term (5 min) accumulation of \(^{13}\)NH₄⁺. In agreement with compartmental analysis, NH₄⁺ fluxes were maximal at 0.1 mM \([K^+]_{\text{ext}}\) (reaching a peak value of 84 \(\mu\)mol g⁻¹ h⁻¹). However, when low-potassium seedlings were exposed to elevated (5 mM) \([K^+]_{\text{ext}}\), NH₄⁺ influx was drastically and immediately reduced, by 26% after the first minute of exposure, and by nearly 50% within 5 minutes (Fig. 4.3).

Sudden increase in \([K^+]_{\text{ext}}\) decreased not only the influx of NH₄⁺, but also its efflux (Fig. 4.4). After introducing an elevated (5 mM) concentration of K⁺ midway through an elution protocol, NH₄⁺ efflux declined notably within a few minutes. Within 15 min following the shift in \([K^+]_{\text{ext}}\), the half-time of cytosolic NH₄⁺ exchange appeared to have been re-established to the value seen prior to the shift (Fig. 4.4). The reverse change in \([K^+]_{\text{ext}}\), from high to low, however, did not immediately elevate NH₄⁺ efflux (Fig. 4.4, inset).

**La³⁺ application mimics K⁺ inhibition of NH₄⁺ influx**

The channel inhibitors TEA, Cs⁺, and La³⁺, which have been shown to reduce channel-mediated fluxes of K⁺ and NH₄⁺ (Wegner et al., 1994; Nielsen and Schjoerring, 1998), were used to help identify the mechanisms underlying the K⁺-sensitive and -insensitive components of NH₄⁺ transport (Fig. 4.5). Interestingly, Cs⁺ and TEA both stimulated NH₄⁺ influx, under high (40 mM) and low (0.1 mM) \([K^+]_{\text{ext}}\), with TEA in particular increasing NH₄⁺ influx by nearly 40%. By contrast, La³⁺ application reduced the influx of NH₄⁺ by 60% under low \([K^+]_{\text{ext}}\) (0.1 mM). At elevated \([K^+]_{\text{ext}}\), no reduction in NH₄⁺ influx was observed. The effects of La³⁺ upon K⁺ influx were also tested, using \(^{42}\)K⁺ as a tracer (Fig. 4.5, inset). Surprisingly, K⁺ influx was
Figure 4.3 Effect of K\(^+\) alterations on NH\(_4\)\(^+\) influx, directly measured using short-term labelling. Barley seedlings were either grown and tested at steady-state K\(^+\) conditions (0.1 or 5 mM [K\(^+\)]\(_{ext}\)), or grown at 0.1 mM [K\(^+\)]\(_{ext}\) but labelled in solution containing 5 mM [K\(^+\)]\(_{ext}\), for 1 or 5 min. All solutions contained 10 mM [NH\(_4\)\(^+\)]\(_{ext}\). Error bars refer to ± SEM of 9 – 17 replicates. Different letters refer to significantly different means (\(P < 0.05\)).
NH$_4^+$ influx (μmol g$^{-1}$ (root FW) h$^{-1}$)

External K$^+$ concentration (mM)

- 0.1 steady state
- 0.1 transition to 5 (1 min)
- 0.1 transition to 5 (5 min)
- 5 steady state

Notes:
- Bar a is significantly different from b, c, and d.
- Bar b is significantly different from c and d.
- Bar c is significantly different from d.
**Figure 4.4** Effect of K\(^+\) alteration on \(^{13}\)NH\(_4^+\) efflux from the roots of intact barley seedlings grown with 10 mM \([\text{NH}_4^+]_{\text{ext}}\) and 0.1 mM \([\text{K}^+]_{\text{ext}}\). The arrow indicates time of shift in \([\text{K}^+]_{\text{ext}}\) from 0.1 to 5 mM. Each point is the mean of 4 – 9 replicates (SEM was, on average, < 9% of the mean), and points prior to the \([\text{K}^+]\) shift are pooled. *Inset.* Reverse effect of K\(^+\) alteration on \(^{13}\)NH\(_4^+\) efflux from the roots of intact barley seedlings grown with 10 mM \([\text{NH}_4^+]_{\text{ext}}\) and 5 mM \([\text{K}^+]_{\text{ext}}\). The arrow indicates time of shift in \([\text{K}^+]_{\text{ext}}\) from 5 to 0.1 mM. Each point is the mean of 2 – 6 replicates (SEM was, on average, < 17% of the mean).
Log \( (^{13}\text{NH}_4)^+ \text{efflux (cpm released g}^{-1}\text{ root FW min}^{-1}) \)

0.1 steady state
○ 0.1 to 5 transition
■ 5 steady state
△ 5 to 0.1 transition

Concentration shift from 0.1 to 5 mM \([K^+]_{ext}\)
Concentration shift from 5 to 0.1 mM \([K^+]_{ext}\)
Figure 4.5 Effect of channel inhibitors on direct NH$_4^+$ influx measurements using short-term labelling. Error bars refer to ± SEM of 6 – 18 replicates. Different letters refer to significantly different means within a [K$^+$]$_{ext}$ ($P < 0.05$). Inset. Direct K$^+$ influx measurements using short-term labelling, in the presence La$^{3+}$ (legend as in larger figure). Error bars refer to ± SEM of 5 – 10 replicates.
NH$_4^+$ influx (µmol g$^{-1}$ (root FW) h$^{-1}$) vs. External K$^+$ concentration (mM)

- Control
- La$^{3+}$
- Cs$^+$
- TEA

K$^+$ influx (µmol g$^{-1}$ (root FW) h$^{-1}$) at External K$^+$ concentration (mM)

- 0.1
- 1.5

Different letters indicate significant differences.
reduced by 70% at the HATS concentration of 0.1 mM, but only by 45% at the LATS concentration of 1.5 mM.

*Active NH$_4^+$ and K$^+$ fluxes can be energetically costly*

We analyzed the energetics of NH$_4^+$ transport using previously reported plasma-membrane electrical potentials from the same plant system (Table 3.1) and cytosolic concentrations of NH$_4^+$ measured in the present study using compartmental analysis (Fig. 4.6). The range of activities presented in Figure 4.6 were determined using activity coefficients corresponding either to a cytosol dominated by K$^+$, NH$_4^+$, and a univalent anion (upper estimate), or to a simple solution of (NH$_4$)$_2$SO$_4$ (lower estimate) (Nobel, 1991; Lide, 2007). Although the cytosolic activities of NH$_4^+$ were significantly different at low and high [K$^+$]$_{ext}$, the electrochemical potential gradient for NH$_4^+$ was inwardly directed in both cases. Thus, NH$_4^+$ influx was determined to occur via facilitated diffusion into the plant cell, and its efflux, in turn, would be energy-demanding.

To test the model and relate it to growth and NH$_4^+$ toxicity, root respiration experiments were conducted (Fig. 4.7). Specific respiratory costs of the active components of K$^+$ and NH$_4^+$ fluxes were determined based on current models of energy usage (Kurimoto et al., 2004) and by use of K$^+$ fluxes determined previously (Fig. 3.3). In all conditions tested, the respiratory costs not associated with NH$_4^+$ and K$^+$ fluxes were similar. The two conditions with the lowest energy requirement for NH$_4^+$ and K$^+$ transport (1.5 and 5 mM K$^+$) had the lowest overall root respiration, but the largest root and shoot masses (Fig. 4.7).
Figure 4.6 Model of NH$_4^+$ unidirectional plasma-membrane fluxes mediated by a low-affinity transport system, and at K$^+$ concentrations representing two K$^+$ influx mechanisms (HATS and LATS). Listed are the directions of active and passive NH$_4^+$ fluxes and measured plasma-membrane electrical potentials ($\Delta$$\Psi$, from Table 3.1). Also included are measured cytosolic NH$_4^+$ concentrations and ranges of NH$_4^+$ activities calculated using two estimates for the ionic strength ($I$) of the cytosol: 1) $I$ based on K$^+$, NH$_4^+$, and a monovalent anion – upper value, or 2) $I$ based on a simple solution of (NH$_4$)$_2$SO$_4$ – lower value.
0.1 mM K⁺

\[ [\text{NH}_4^+] = 10 \text{ mM} \]
\( (a_{\text{NH}_4} = 7.6 \text{ mM}) \)

\[ [\text{NH}_4^+] = 935 \text{ mM} \]
\( (a_{\text{NH}_4} = 240 - 580 \text{ mM}) \)
\[ \Delta \Psi_{PM} = -120 \text{ mV} \]

Facilitated diffusion

Active (pump)

5 mM K⁺

\[ [\text{NH}_4^+] = 10 \text{ mM} \]
\( (a_{\text{NH}_4} = 7.6 \text{ mM}) \)

\[ [\text{NH}_4^+] = 200 \text{ mM} \]
\( (a_{\text{NH}_4} = 90 - 150 \text{ mM}) \)
\[ \Delta \Psi_{PM} = -120 \text{ mV} \]

Facilitated diffusion

Active (pump)
Figure 4.7 Measured rate of oxygen uptake and whole plant fresh weights for individual barley seedlings. Each bar (oxygen uptake) has been divided into the amount of respiration necessary to drive $\text{K}^+$ or $\text{NH}_4^+$ influx, and for maintenance and growth. Error bars refer to $\pm$ SEM of 12 – 14 replicates. Each point (whole plant FW) refers to the sum of roots + shoots. Error bars refer to $\pm$ SEM of 90 – 270 individual seedlings. Different letters refer to significantly different means: a-b (oxygen uptake); c-f (whole plant FW) ($P < 0.05$).
O₂ consumption (µmol g⁻¹ (root FW) h⁻¹)

Total plant FW (g plant⁻¹)

External K⁺ concentration (mM)

Other costs

NH₄⁺ flux

K⁺ flux

Tissue FW

0.1 1.5 5 40
Discussion

Rapid, futile $\text{NH}_4^+$ cycling

Efflux of ions from plant cells into the external environment can be monitored using several techniques, but none is as comprehensive as compartmental analysis by tracer efflux, which facilitates the simultaneous measurement of unidirectional fluxes, subcellular concentrations, and compartmental exchange rates of labelled ions (Lee and Clarkson, 1986; Siddiqi et al., 1991; Britto et al., 2001, Kronzucker et al., 2003b). This technique initially led to the discovery of rapid, futile cellular $\text{NH}_4^+$ cycling in a number of plant systems (Britto et al., 2001, 2002; Kronzucker et al., 2003a; Britto and Kronzucker, 2006), and to the close association of this phenomenon with $\text{NH}_4^+$ toxicity (Kronzucker et al., 2001). In the present study, we confirmed the high unidirectional fluxes of $\text{NH}_4^+$ in both directions across the plasma membrane of barley root cells, as well as the high ratio of efflux to influx, both of which are key characteristics of the futile cycling condition (Fig. 4.2).

Because $\text{NH}_4^+$ toxicity in plants can be relieved by increasing potassium availability (Cao et al., 1993; Spalding et al., 1999; Santa-Maria et al., 2000; Kronzucker et al., 2003b; see Chapter 3), it was hypothesized that changes in external $[\text{K}^+]$ would alter the magnitude of $\text{NH}_4^+$ fluxes and the degree of futile cycling in $\text{NH}_4^+$-susceptible barley plants. As shown in Figure 4.2, this hypothesis was borne out on both counts. Changing the steady-state $\text{K}^+$ supply from 0.1 mM to 1.5 mM significantly reduced both influx and efflux of $\text{NH}_4^+$, and substantially decreased the ratio of efflux to influx. Increasing $[\text{K}^+]_{\text{ext}}$ to higher values (5 and 40 mM) had no further effect on these flux parameters. Importantly, this shift is also associated with relief from $\text{NH}_4^+$ toxicity (Fig. 4.7). Increasing $[\text{K}^+]_{\text{ext}}$ from 0.1 to 1.5 mM or above caused a reduction in both root tissue
and cytosolic NH$_4^+$ of approximately 70%, showing that the amelioration of NH$_4^+$ toxicity by increasing [K$^+$]$_{ext}$ was paralleled by a reduction in NH$_4^+$ tissue content (Fig. 4.6, Table 4.1). Our [NH$_4^+$]$_{cyt}$ values for the toxic condition, while high, are in agreement with values reported in a separate study on NH$_4^+$ toxicity in barley seedlings (Britto et al., 2001). Moreover, our tissue NH$_4^+$ values are well in excess of what is required to account for the [NH$_4^+$]$_{cyt}$ estimates, demonstrating that a substantial NH$_4^+$ pool is also present in the vacuole. While vacuolar pools were not directly measured here, since the focus of the study was the futile cycling of NH$_4^+$ at the plasma membrane, subtraction of cytosolic content from whole-root content of NH$_4^+$ yields estimates of vacuolar pools that range between 2.4 and 25 μmol g$^{-1}$(FW) (depending on N and K status; not shown), in good agreement with prior studies (Lee and Ratcliffe, 1991; Wang et al., 1993).

While the dramatic differences in key flux parameters shown in Figure 4.2 were determined under steady-state nutritional conditions, it was of further interest to examine the timescale over which K$^+$-induced alterations in NH$_4^+$ transport occur. Figures 4.3 and 4.4 show that these potent effects become manifest within 1 to 5 minutes of increased K$^+$ supply, for both influx and efflux of NH$_4^+$. The rapidity of this response suggests that K$^+$ regulates NH$_4^+$ fluxes possibly by acting allosterically on an ammonium transporter, or by competing directly with NH$_4^+$ for a common transport mechanism. Such short-term changes are likely to precede longer-term changes that involve alterations in gene expression, that would bring about the further lowering of NH$_4^+$ influx seen in the rightmost column in Figure 4.3. The lack of an immediate stimulatory effect on NH$_4^+$ influx by a reduction in external [K$^+$] may indicate that the shift from the high-K$^+$ condition to the low-K$^+$ condition entails the upregulation of NH$_4^+$-sensitive transporters, which would occur over a longer time scale. Alternatively or in addition, it may
indicate that the release of K\(^+\) from inhibitory binding sites on NH\(_4^+\) transporters also occurs over a longer time scale.

\textit{K\(^+\)-sensitive NH\(_4^+\) influx pathway}

It is not yet fully resolved how NH\(_4^+\) enters the plant cell, particularly in the low-affinity range. Our study provides new insight into possible candidates and characteristics of low-affinity NH\(_4^+\) transport. The dramatic reduction of NH\(_4^+\) fluxes and cycling brought about by elevating [K\(^+\)]\(_{\text{ext}}\) from 0.1 to 1.5 mM or higher (Fig. 4.2), is strong evidence that at least two pathways of NH\(_4^+\) influx operate simultaneously, one sensitive and the other insensitive to [K\(^+\)]\(_{\text{ext}}\). Because NH\(_4^+\) influx under LATS conditions is high-capacity and energetically passive (Fig. 4.6; also see section on energetics below), it is very likely that ion channels are responsible for catalyzing both components of the flux.

More specifically, our evidence suggests that the K\(^+\)-sensitive pathway, which catalyzes the greater amount of NH\(_4^+\) influx, involves the operation of either nonselective cation channels or inward rectifying K\(^+\) channels, such as AKT1. NSCCs comprise a large group of relatively uncharacterized transporters that have been shown to transport a variety of ions, including Na\(^+\), Ca\(^{2+}\), K\(^+\), and NH\(_4^+\) (Demidchik et al., 2002b). NSCCs are inhibited by lanthanides, but tend to be insensitive to traditional K\(^+\) channel blockers, particularly Cs\(^+\) and TEA (Tyerman and Skerrett, 1999). In the present study, NH\(_4^+\) influx at 0.1 mM [K\(^+\)]\(_{\text{ext}}\) was strongly inhibited by the relatively broad-spectrum channel blocker La\(^{3+}\), but no La\(^{3+}\) effect was observed on the K\(^+\)-suppressed flux at 40 mM [K\(^+\)]\(_{\text{ext}}\) (Fig. 4.5). This is consistent with the idea that, at this supply level, K\(^+\) much more effectively competes for a La\(^{3+}\)-sensitive NSCC pathway, making it unavailable for NH\(_4^+\) transport. Evidence that K\(^+\) is indeed also transported through the La\(^{3+}\)-
sensitive pathway is seen in the inhibitory effect of La$^{3+}$ on K$^+$ influx (Fig. 4.5, inset; the inhibitory effect on the low-K$^+$ control is most likely due to the suppression of high-affinity KUP/HAK/KT transporters by NH$_4^+$; see Spalding et al., 1999, and Introduction). The lack of inhibition of NH$_4^+$ influx by Cs$^+$ and TEA is also consistent with permeation through NSCCs. Indeed, it is tempting to attribute K$^+$-sensitive, La$^{3+}$-sensitive NH$_4^+$ transport to the activity of the weakly voltage-dependent NSCC described by White and Lemtiri-Chlieh (1995), White (1996a), and Davenport and Tester (2002), because this channel displays several physiological attributes strongly reminiscent of the characteristics defined here: transport of both NH$_4^+$ and K$^+$ as competing substrates; relative insensitivity to TEA and Cs$^+$; and strong inhibition by the lanthanide Gd$^{3+}$ as well as by La$^{3+}$ itself. The observation that, in our study, TEA and Cs$^+$ actually stimulated NH$_4^+$ influx at both K$^+$ conditions tested (Fig. 4.5) is perhaps surprising, but supports the finding that both of these agents can increase Na$^+$ influx (Wang et al., 2006), and TEA has also been shown to increase the influx of both Ca$^{2+}$ (Demidchik et al., 2002a) and Cs$^+$ (Hampton et al., 2004) in roots of Arabidopsis. In addition, both of the latter studies postulated that the transporters involved were NSCCs, and showed that Gd$^{3+}$ was effective in inhibiting the TEA-stimulated fluxes, a result very similar to the La$^{3+}$ suppression of TEA-stimulated NH$_4^+$ influx observed in the present study.

Many studies, in a variety of organisms including bacteria, yeast, animals, and plants, have suggested that NH$_4^+$ enters the cell through K$^+$-specific channels (Wang et al., 1996; Nielsen and Schjoerring, 1998; Hess et al., 2006), and the K$^+$-suppression of NH$_4^+$ influx (Fig. 4.2) supports the idea that K$^+$ channels are responsible for the K$^+$-sensitive component of low-affinity NH$_4^+$ uptake, instead of, or in addition to, NSCCs. At higher [K$^+$]$_{ext}$, these channels would be occupied by K$^+$, limiting NH$_4^+$ influx to NH$_4^+$-specific pathways. Consistent with this
observation was our finding that La$^{3+}$ (known to block K$^+$ channels as well as NSCCs; Wegner et al., 1994) blocked NH$_4^+$ influx at low [K$^+$]$_{ext}$, in addition to reducing K$^+$ influx at both high and low [K$^+$]$_{ext}$ (Fig. 4.5). However, the stimulation of NH$_4^+$ influx by the K$^+$-channel blockers Cs$^+$ and TEA does not support NH$_4^+$ permeation through K$^+$ channels. Thus, the proposal that NSCCs are responsible for the K$^+$-sensitive component of root NH$_4^+$ influx in the LATS range is more congruent with our data. Low-affinity fluxes in shoots may be mediated by a different mechanism, as was shown by Nielsen and Schjoerring (1998), who observed in leaves of *Brassica napus* a 30% and 47% reduction in NH$_4^+$ influx with La$^{3+}$ and Cs$^+$ treatments, respectively.

*K$^+$-insensitive NH$_4^+$ influx pathway*

In addition to K$^+$-sensitive NH$_4^+$ conductance, a substantial portion of low-affinity NH$_4^+$ entry into barley root cells is mediated by a K$^+$-insensitive mechanism (Fig. 4.2). This mechanism is resistant to increases in [K$^+$]$_{ext}$ from 1.5 to 40 mM, suggesting that, because of the lack of a competitive effect, the transporter involved is neither a K$^+$-specific channel nor an NSCC. Several other possibilities arise as to its molecular identity. One is that it is a high-affinity NH$_4^+$ transporter, such as AMT1 (Ninnemann et al., 1994; Rawat et al., 1999), which may have some dual-affinity character, such as has been seen for nitrate and potassium transporters (Fu and Luan, 1998; Liu et al., 1999). However, AMT1-mediated NH$_4^+$ transport is downregulated by high NH$_4^+$, both genetically and functionally (Rawat et al., 1999), which eliminates its likelihood as a candidate for NH$_4^+$ influx under the NH$_4^+$ supply (10 mM) used in our study. Another possibility is that NH$_4^+$ permeates via aquaporins. Several recent studies have shown that in addition to water, *Xenopus* oocytes expressing *Arabidopsis* TIP genes (encoding aquaporins)
could mediate the transport of small molecules such as CO$_2$, glycerol, urea, NH$_4^+$, and NH$_3$ (Uehlein et al., 2007). However, Detmers et al. (2006) found that TEA effectively inhibited aquaporin-mediated water transport, while in our study TEA failed to inhibit NH$_4^+$ influx, casting doubt on the role of aquaporins in low-affinity NH$_4^+$ transport. The elimination of these two candidates suggests that NH$_4^+$ enters root cells under high-K$^+$, high-NH$_4^+$ conditions via NH$_4^+$-specific channels, the molecular identity of which remains to be determined.

**Energetics of NH$_4^+$ and K$^+$ unidirectional fluxes**

It is instructive to examine, from an energetics perspective, the unidirectional NH$_4^+$ fluxes observed here. Our thermodynamic analysis shows that, under all experimental conditions, NH$_4^+$ exchange across the plasma membrane takes the form of a “leak-pump” scenario, i.e. with passive NH$_4^+$ influx coupled to active NH$_4^+$ efflux (Fig. 4.6). In this respect, bidirectional NH$_4^+$ transport follows a pattern that has been observed in the low-affinity exchange of other major cations, such as K$^+$ (Table 3.1) and Na$^+$ (Wang et al., 2006; Kronzucker et al., 2006). Based upon current models of ion transport (Kurimoto et al., 2004; Britto and Kronzucker, 2006), which consider the coupling and stoichiometry of ion fluxes in relation to proton fluxes and ATP hydrolysis, we estimated the respiratory cost of NH$_4^+$ and K$^+$ transport, and compared them to measured respiration rates and growth (Fig. 4.7). In agreement with estimates indicating that, under certain conditions, as much as 70% of total root respiration can be invested in the transport of the NO$_3^-$ anion (Scheurwater et al., 1999), we found that as much as 64% of our measured respiration rates could be accounted for by the combined plasma-membrane fluxes of the two cations NH$_4^+$ and K$^+$. The plants that displayed rapid, futile cycling of NH$_4^+$ or K$^+$ not only showed the highest respiration, but had significant reductions in total plant biomass (Fig. 4.7).
We attribute this effect to the differential allocation of carbohydrate supply in the various treatments, with a greater proportion directed towards the wasteful process of futile ion cycling, in the case of the growth-compromised plants.

Concluding Remarks

This study provides the first demonstration of the parallel operation of K$^+$-sensitive and -insensitive root NH$_4^+$ fluxes in the low-affinity transport range, and offers insight into the mechanism by which K$^+$ is able to alleviate NH$_4^+$ toxicity. Elevated K$^+$ eliminates a major fraction of low-affinity NH$_4^+$ influx, and substantially reduces the amount of futile cycling of this toxic ion. Intriguingly, this effect contrasts sharply with the effect of NH$_4^+$ on K$^+$ transport, where high-affinity influx is diminished by NH$_4^+$, but low-affinity influx remains unaffected (Fig. 3.3; Spalding et al., 1999; Kronzucker et al., 2003b). We propose that low-affinity NH$_4^+$ transport may be mediated by the dual operation of non-selective, K$^+$-sensitive cation channels on the one hand, and K$^+$-insensitive, NH$_4^+$-specific channels on the other. However, we should point out that our study does not rule out the existence of other mechanisms of low-affinity NH$_4^+$ transport, in addition to the two proposed here. The shift between low and high external [K$^+$] steady states may entail the expression of a genetically and mechanistically distinct complement of transporters, a possibility that only extensive new genetic analyses can unravel. Nevertheless, the physiological observations presented here, that: 1) the NH$_4^+$ fluxes under both high and low [K$^+$] conditions show virtually the same degree of La$^{3+}$ resistance (Fig. 4.5); and that 2) NH$_4^+$ influx is rapidly suppressed, when the low-K$^+$ condition is suddenly altered to a high-K$^+$ condition, almost to the same extent as observed at a high-K$^+$ steady-state, strongly suggest that the La$^{3+}$- and K$^+$-insensitive component of low-affinity NH$_4^+$ influx is operative under all K$^+$
conditions. Our study demonstrates how pivotal a role K$^+$ plays in the regulation of NH$_4^+$ toxicity, reducing the energy burden of toxic NH$_4^+$ fluxes, and substantially improving growth under a high NH$_4^+$ nutritional regime.
CHAPTER 5:

NH$_4^+$-stimulated and -inhibited components of K$^+$ transport in rice

(*Oryza sativa L.*)

A modified version of this chapter will be submitted for review as:


*Dr. Kronzucker contributed substantially to the formulation of ideas for this manuscript, as well as to the writing, and data discussions

*Dr. Britto contributed substantially to the writing and to the data discussions

*Amanda Ali assisted with data collection*
Abstract

The disruption of K$^+$ transport and accumulation is symptomatic of NH$_4^+$ toxicity in plants. In this study, we examined the influence of K$^+$ supply (0.02 – 40 mM) and nitrogen source (10 mM NH$_4^+$ or NO$_3^-$) on root plasma-membrane K$^+$ fluxes and cytosolic K$^+$ pools, plant growth, and whole-plant K$^+$ distribution in the NH$_4^+$-tolerant plant species rice (Oryza sativa L.). Using the radiotracer $^{42}$K$^+$, tissue mineral analysis, and growth data, we show that rice is affected by NH$_4^+$ toxicity under high-affinity K$^+$ transport conditions. Substantial recovery of growth was seen as [K$^+$]$_{ext}$ was increased from 0.02 to 0.1 mM, and, at 1.5 mM, growth was superior on NH$_4^+$. Growth recovery at these concentrations was accompanied by greater influx of K$^+$ into root cells, translocation of K$^+$ to the shoot, and tissue K$^+$. In the low-affinity K$^+$ transport range, NH$_4^+$ stimulated K$^+$ influx relative to NO$_3^-$ controls. We conclude that rice, despite its well-known tolerance to NH$_4^+$, nevertheless displays considerable growth suppression and disruption of K$^+$ homeostasis under this N regime at low [K$^+$]$_{ext}$, but displays efficient recovery from NH$_4^+$ inhibition, and indeed a stimulation of K$^+$ acquisition, when [K$^+$]$_{ext}$ is increased.

Introduction

Maintenance of potassium (K$^+$) homeostasis is critical to plant cell function. However, the uptake of K$^+$ and its distribution within the plant varies widely with environmental conditions. One of the chief factors influencing plant-potassium relations is the chemical speciation of inorganic nitrogen (N) in soil. In particular, ammonium (NH$_4^+$) has been shown to reduce the primary influx of K$^+$ from the external environment, and suppress its accumulation in
plant tissues (Kirkby and Mengel, 1967; Scherer et al., 1984; Vale et al., 1987, 1988; Van Beusichem et al., 1988; Engels and Marschner, 1993; Peuke and Jeschke, 1993; Wang et al., 1996; Gerendás et al., 1997; Santa-María et al., 2000; Bañuelos et al., 2002; Kronzucker et al., 2003b). This is a key feature of NH$_4^+$ toxicity, which affects the majority of plant species when exposed to elevated soil concentrations of NH$_4^+$ (typically, when [NH$_4^+$] > 1 mM; Britto et al., 2001, 2002; Britto and Kronzucker, 2002). However, the NH$_4^+$-dependent inhibition of K$^+$ influx and accumulation can be alleviated by increasing the external K$^+$ concentration ([K$^+$]$_{ext}$; Cao et al., 1993; Spalding et al., 1999; Santa-María et al., 2000; Kronzucker et al., 2003b). The sensitivity of K$^+$ influx to NH$_4^+$ appears to depend on the mechanism of primary K$^+$ uptake that dominates at a given [K$^+$]$_{ext}$: at micromolar concentrations, K$^+$ uptake is mainly mediated by NH$_4^+$-suppressible, high-affinity transport system (HATS), while at higher, millimolar [K$^+$]$_{ext}$, K$^+$ influx is mediated by an NH$_4^+$-resistant, low-affinity transport system (LATS) (Spalding et al., 1999; Santa-María et al., 2000; Kronzucker et al., 2003b). The precise mechanism by which NH$_4^+$ inhibits high-affinity K$^+$ influx has not been elucidated, although it has been suggested that NH$_4^+$ competitively inhibits K$^+$ transport at the protein level (Vale et al., 1987; Wang et al., 1996).

In ammonium-sensitive barley (*Hordeum vulgare* L.), NH$_4^+$ has been shown to disrupt not only the primary influx, but also the internal distribution of K$^+$ at both whole-plant and cellular levels. For example, Santa-María et al. (2000) and Kronzucker et al. (2003b) found that NH$_4^+$ reduced K$^+$ translocation from root to shoot by 60% to 90%. At a subcellular level, radiotracer studies have shown that cytosolic [K$^+$] is suppressed by high [NH$_4^+$]$_{ext}$ (Kronzucker et al., 2003b). The disruption of K$^+$ translocation and cytosolic K$^+$ homeostasis are, most likely, related: while NH$_4^+$ is not transported in large amounts to the shoot (Kronzucker et al., 1998;
Husted et al., 2000), its effect on cytosolic [K+] or upon K+ translocation pathways in the root may play a critical role in NH4+ sensitivity by reducing the xylem loading of K+ (Gaymard et al., 1998; Johansson et al., 2006; Liu et al., 2006).

Rice (Oryza sativa L.), one of the world’s most important crop species, displays greater tolerance to NH4+ than other cereals (Sasakawa and Yamamoto, 1978). Given the pivotal role of K+ nutrition in the development of NH4+ toxicity or tolerance, it was therefore important to investigate the degree to which rice plants may be able to resist NH4+-induced disruptions in primary K+ acquisition, cellular K+ homeostasis, and root-to-shoot K+ translocation. These disruptions have been characterized in barley and other NH4+-sensitive plant species, but have not been examined in detail in NH4+-tolerant plant species. Here, we have conducted compartmental analyses using the radiotracer 42K+ to evaluate K+ transport and compartmentation in intact seedlings of NH4+-tolerant rice, examining plant performance at four levels of K+ supply (0.02-40 mM, spanning the high- and low-affinity transport ranges), with either NH4+ or nitrate (NO3−) as the sole N source (10 mM). We hypothesized that K+ transport and distribution, at whole-plant and subcellular levels, would resist disruption by NH4+ provision, in ammonium-tolerant rice.

Material and Methods

Plant Culture

Rice seeds (Oryza sativa L. cv. ‘IR-72’) were surface-sterilized for 10 min in 1% sodium hypochlorite, and germinated in water for 2 d prior to placement in 4-L vessels containing aerated, modified Johnson’s solution (2 mM MgSO4; 1 mM CaCl2; 0.3 mM NaH2PO4; 0.1 mM
Fe-EDTA; 20 μM H₃BO₃; 9 μM MnCl₂; 1.5 μM CuSO₄; 1.5 μM ZnSO₄; 0.5 μM Na₂MoO₄), pH 6 – 6.5, for an additional 19 d. The growth solutions were modified to provide four concentrations of potassium (as K₂SO₄), at 0.02, 0.1, 1.5, and 40 mM, and nitrogen (10 mM) as either (NH₄)₂SO₄, or Ca(NO₃)₂. Solutions were exchanged frequently to ensure that plants remained at a nutritional steady state, and to ensure that solution pH was maintained between 6 and 6.5. Solutions were exchanged on day (with the first two days spent in water for germination): 8; 12; 15; 17; 19; 20. Plants were cultured in climate-controlled walk-in growth chambers under fluorescent lights, providing a tropical environment for the rice seedlings, with a day/night temperature cycle of 30°C/ 20°C, an irradiation of 425 μmol photons m⁻² s⁻¹ at plant height for 12 h d⁻¹ (Sylvania Cool White, F96T12/CW/VHO), and a relative humidity of 70%. On day 19 (2 d prior to experimentation), seedlings were bundled together in groups of 3-5 at the stem base using a plastic collar, 0.5 cm in height.

Steady-state influx, translocation, and pool size measurements

Plasma-membrane fluxes, cytosolic pool sizes, and shoot translocation of K⁺ were determined under steady-state conditions using compartmental analysis by tracer efflux (for details see Appendix I; Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995, 2003b). Briefly, intact roots of seedlings were labelled for 60 min in a solution identical to the growth solution except that it contained the radiotracer ⁴²K⁺ (t½ = 12.36 h, provided by McMaster University Nuclear Reactor, Hamilton, Ontario, Canada). Labelled seedlings were then attached to efflux funnels and eluted of radioactivity for 30 min, using a timed series (15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once), and 2 min (eight times); see Fig. 2) of non-
radioactive desorption solutions (as 13 or 20-mL aliquots), identical to the growth solutions. All solutions were mixed using a fine stream of air bubbles. After elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s, and fresh weights were determined. Radioactivity from eluates, roots, and shoots was measured by gamma counting (Perkin-Elmer Wallac 1480 Wizard 3”, Turku, Finland, or Canberra-Packard, Quantum Cobra Series II, Model 5003).

Exponentially declining rates of $^{42}$K$^+$ release from roots over time were then analyzed using linear regression (see Fig. 5.2). The function $\ln \varphi_{co(t)}^* = \ln \varphi_{co(i)}^* – kt$ (in which $\varphi_{co(t)}^*$ is tracer efflux at elution time $t$, $\varphi_{co(i)}^*$ is initial tracer efflux, and $k$, found from the slope of the changing tracer release rate, is the rate constant describing the exponential decline in tracer efflux) was used to resolve the kinetics of the slowest-exchanging phase, which represents tracer exchange with the cytosolic compartment (Behl and Jeschke, 1981; Memon et al., 1985a; Kronzucker et al., 2003b). Chemical efflux, $\varphi_{co}$, was determined from $\varphi_{co(i)}^*$, divided by the specific activity of the cytosol ($S_c$) at the end of the labelling period (this activity was determined using the exponential-rise function $S_c = S_o (1 – e^{-kt})$, in which $S_o$ is the specific activity of the external solution, $t$ is labelling time, and $k$ is as described above). Net flux, $\varphi_{net}$, was found using total-plant $^{42}$K$^+$ retention after desorption. Influx, $\varphi_{oc}$, was calculated from the sum of $\varphi_{net}$ and $\varphi_{co}$. Translocation of $^{42}$K$^+$ to the shoot was determined by normalizing tracer accumulation at the end of the elution protocol for the specific activity of the loading solution and by the root fresh weight. Cytosolic [K$^+$] ([K$^+$]$_{cyt}$) was determined using the flux-turnover equation, $[K^+]_{cyt} = \Omega \cdot \varphi_{oc} / k$, where $\Omega$ is a proportionality constant correcting for the cytosolic volume being approximately 5% of total tissue (Lee and Clarkson, 1986; Siddiqi et al., 1991).
Short-term non-steady-state influx measurements

To examine the effect changing $[\text{K}^+]_{\text{ext}}$ on $\text{K}^+$ influx, unidirectional influx of $\text{K}^+$ under non-steady-state conditions was determined directly using short-term labelling with $^{42}\text{K}^+$ (see Appendix I; Britto and Kronzucker, 2001). Seedlings grown at 0.1 mM $[\text{K}^+]_{\text{ext}}$ were pre-equilibrated for 5 min in growth solution, then immersed in labelling solution for another 5 min. This solution was identical to the growth solution, except that it contained $^{42}\text{K}^+$ for a final $[\text{K}^+]_{\text{ext}}$ between 0.1 and 5 mM. Plants were then transferred to a non-radioactive solution for 5 s to reduce tracer carryover to the desorption solution, and finally desorbed for 5 min in fresh nutrient solution.

Tissue $\text{K}^+$ content

To measure tissue $\text{K}^+$ content, roots of rice seedlings were first desorbed for 5 min in 10 mM CaSO$_4$ to remove extracellular $\text{K}^+$. Roots and shoots were then separated and weighed. Tissue was oven dried for a minimum of 72 h at 80 – 85°C, reweighed, pulverized, and digested with 30% HNO$_3$ for a minimum of 72 h. $\text{K}^+$ concentrations in tissue digests were determined using a single-channel flame photometer (Digital Flame Analyzer model 2655-00, Cole-Parmer, Anjou, Quebec, Canada).

Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons meeting the assumptions of the Dunnett’s C exam, (not assuming equal variances), with the statistical package SPSS (ver. 12).
Results

At the lowest external K\(^+\) supply of 0.02 mM, growth of rice seedlings was suppressed by about 50% when nitrogen was supplied as NH\(_4^+\), relative to NO\(_3^-\) controls (Table 5.1). Growth on NH\(_4^+\) was also significantly lower at 0.1 mM [K\(^+\)]\(_{\text{ext}}\), although to a much lesser extent (fresh weight was diminished by only 10%). At higher levels of K\(^+\) supply, NH\(_4^+\) either increased fresh weight (by nearly 50% at 1.5 mM [K\(^+\)]\(_{\text{ext}}\)), or had no significant effect relative to NO\(_3^-\) (at 40 mM). Maximal growth with NH\(_4^+\) as sole N source was observed at 1.5 mM [K\(^+\)]\(_{\text{ext}}\), rather than at the highest provision of 40 mM, at which suboptimal growth occurred.

The growth trends shown in Table 5.1 were reflected in the K\(^+\) content of roots and shoots (Fig. 5.1). At the lowest values of [K\(^-\)]\(_{\text{ext}}\) (0.02 and 0.1 mM), tissue K\(^+\) accumulation was strongly inhibited by NH\(_4^+\) relative to NO\(_3^-\), in both roots and shoots. At 1.5 and 40 mM [K\(^+\)]\(_{\text{ext}}\), this relative inhibition was reversed in shoots, with NH\(_4^+\)-grown seedlings accumulating between 25 and 40% more K\(^+\) than found in NO\(_3^-\)-grown plants.

Compartmental analysis with the radiotracer $^{42}\text{K}^+$ was used to compare the influence of NH\(_4^+\) and NO\(_3^-\) nutrition on subcellular K\(^+\) fluxes and cytosolic K\(^+\) compartmentation in the rice seedlings (Fig. 5.2). Unidirectional influx of K\(^+\) across the plasma membrane of root cells generally increased with increasing [K\(^+\)]\(_{\text{ext}}\), and a strong influence of N source on this flux was observed (Fig. 5.3). At the lowest values of [K\(^-\)]\(_{\text{ext}}\) (0.02 and 0.1 mM), K\(^+\) influx was significantly inhibited with NH\(_4^+\) nutrition in rice, paralleling the inhibition of growth and K\(^+\) accumulation in tissue. At 1.5 mM [K\(^+\)]\(_{\text{ext}}\), no difference was seen in K\(^+\) influx in seedlings grown with either NH\(_4^+\) or NO\(_3^-\), while, surprisingly, at the highest [K\(^+\)]\(_{\text{ext}}\) value of 40 mM, influx was stimulated by NH\(_4^+\) provision.
Table 5.1 Tissue fresh weight (root + shoot) for 3-week rice seedlings (shoot fresh weights are shown in parentheses). Error bars refer to ± SEM (n ≥ 5 replicates). Asterisks indicate significantly higher means between N treatments for each K⁺ condition examined, with $P < 0.05$. 
<table>
<thead>
<tr>
<th>$[\text{K}^+]_{\text{ext}}$</th>
<th>NO$_3^-$ treatment</th>
<th>NH$_4^+$ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>109 ± 10 (55 ± 6)</td>
<td>52 ± 4 (33 ± 2)</td>
</tr>
<tr>
<td>0.1</td>
<td>300 ± 7 (170 ± 4)</td>
<td>267 ± 5 (172 ± 3)</td>
</tr>
<tr>
<td>1.5</td>
<td>251 ± 27 (134 ± 16)</td>
<td>367 ± 31 (210 ± 18)</td>
</tr>
<tr>
<td>40</td>
<td>244 ± 33 (128 ± 17)</td>
<td>220 ± 23 (130 ± 15)</td>
</tr>
</tbody>
</table>
Figure 5.1 K⁺ tissue content of rice seedlings grown at four external K⁺ concentrations (0.02, 0.1, 1.5, and 40 mM). Error bars refer to ± SEM of 6 – 18 replicates, with asterisks indicating significantly different means between N treatments (NO₃⁻ or NH₄⁺) for each K⁺ condition and plant organ (root or shoot) examined ($P < 0.05$).
The figure shows the potassium (K⁺) content (μmol g⁻¹ (root or shoot FW)) in various treatments as a function of external K⁺ concentration (mM). The treatments include NH₄⁺-grown root, NO₃⁻-grown root, NH₄⁺-grown shoot, and NO₃⁻-grown shoot. The graph indicates a significant increase in K⁺ content with increasing external K⁺ concentration, particularly at the higher concentrations of 1.5 and 40 mM.
**Figure 5.2** $^{42}\text{K}^+$ efflux pattern in roots of rice seedlings grown at 0.1 mM K$^+$ and 10 mM NH$_4^+$.

Shown is the entire data set ($n = 15$) for this treatment, illustrating the reproducibility of the data. The solid line represents averaged $^{42}\text{K}^+$ release from the cytosol. SEM for each point was within 10% of the mean.
Log ($\log_{10}(\text{K}^+ \text{efflux (cpm released g}^{-1}\text{root FW min}^{-1})$))

Elution time (min)
Figure 5.3 Steady-state influx of K\(^+\) in roots of rice at 10 mM NO\(_3^-\) or NH\(_4^+\), and at four external [K\(^+\)]. Error bars refer to ± SEM of 5 – 15 replicates, with asterisks indicating significantly different means between N treatments for each K\(^+\) condition (P < 0.05).
$K^+$ influx (μmol g$^{-1}$ (root FW) h$^{-1}$)

- **NH$_4$**$^+$-grown
- **NO$_3$**$^-$-grown

**External $K^+$ concentration (mM)**

- 0.02
- 0.1
- 1.5
- 40

* indicates statistical significance.
Figure 5.4 shows cytosolic concentrations of K\(^+\) ([K\(^+\)\(_{\text{cyt}}\)]) for roots of rice seedlings, over the range of tested conditions. Again, a strong interaction between K and N nutrition was observed: at the same values of low [K\(^+\)\(_{\text{ext}}\)] and high NH\(_4^+\) that brought about growth inhibition, tissue-K\(^+\) suppression, and lower influx of K\(^+\), there was a significant decline in [K\(^+\)\(_{\text{cyt}}\)] in roots of rice seedlings. This trend was not seen at higher [K\(^+\)\(_{\text{ext}}\)]; on the contrary, at the highest [K\(^+\)\(_{\text{ext}}\)], cytosolic K\(^+\) pools of rice were larger under NH\(_4^+\) nutrition. Interestingly, increasing [K\(^+\)\(_{\text{ext}}\)] from the HATS-range value of 0.1 mM to the LATS-range value of 1.5 mM resulted in a lowering of [K\(^+\)\(_{\text{cyt}}\)] under steady-state conditions, regardless of the N source.

Figure 5.5 illustrates the effect of N source on \(^{42}\)K\(^+\) transport to the shoot in rice seedlings. Rice seedlings showed suppression of \(^{42}\)K\(^+\) translocation at the lowest [K\(^+\)\(_{\text{ext}}\)] values (0.02 and 0.1 mM), with a maximum 65% reduction at the lowest K\(^+\) condition. At higher [K\(^+\)\(_{\text{ext}}\)] (1.5 and 40 mM), NH\(_4^+\)-grown rice displayed substantially (as much as 90%) greater translocation of \(^{42}\)K\(^+\), compared to NO\(_3^-\) controls.

Figure 5.6 shows the influx of K\(^+\) into rice seedlings, as determined by short-term (5 min) accumulation of \(^{42}\)K\(^+\). In agreement with compartmental analysis, K\(^+\) influx at low (0.1 mM [K\(^+\)\(_{\text{ext}}\)]) was reduced in NH\(_4^+\)-grown seedlings relative to those grown with NO\(_3^-\). Non-steady-state influx experiments, in which seedlings grown at low [K\(^+\)\(_{\text{ext}}\)] were transiently exposed to elevated (between 0.1 and 5 mM) [K\(^+\)\(_{\text{ext}}\)], showed that K\(^+\) influx increased significantly with increased substrate, regardless of N condition. However, K\(^+\) influx was the highest in NH\(_4^+\)-grown seedlings following the change in [K\(^+\)\(_{\text{ext}}\)], with K\(^+\) influx increasing by 5 – 6.5 times, as compared to NO\(_3^-\)-grown seedlings, in which influx only doubled.
Figure 5.4 Cytosolic $K^+$ concentrations (mM) as determined by compartmental analysis in roots of rice seedlings grown at 10 mM $\text{NO}_3^-$ or $\text{NH}_4^+$, and at four external $[K^+]$. Error bars refer to ± SEM of 5 – 15 replicates, with asterisks indicating significantly different means between N treatments for each $K^+$ condition ($P < 0.05$).
Cytosolic K$^+$ concentration (mM) vs. External K$^+$ concentration (mM)

- **NH$_4^+$-grown**
- **NO$_3^-$-grown**

* indicates a significant difference.
Figure 5.5 Shoot accumulation of $^{42}\text{K}^+$ following labelling of rice seedlings grown at 10 mM NO$_3^-$ or NH$_4^+$, and at four external [K$^+$]. Error bars refer to ± SEM of 5 – 15 replicates, with asterisks indicating significantly different means between N treatments for each K$^+$ condition ($P < 0.05$).
$^{42}\text{K}^+$ shoot content (cpm g$^{-1}$ (root FW) x 10$^6$)

- **NH$^+_4$-grown**
- **NO$_3^-$-grown**

External $\text{K}^+$ concentration (mM)
Figure 5.6 Effect of changing external [K$^+$] on K$^+$ influx, measured directly using short-term labelling. Rice seedlings were grown at 0.1 mM [K$^+$]$_{\text{ext}}$, and either 10 mM [NO$_3^-$]$_{\text{ext}}$ (open circles) or 10 mM [NH$_4^+$]$_{\text{ext}}$ (black circles), and labelled in solutions spanning 0.1 to 5 mM [K$^+$]$_{\text{ext}}$ for 5 min. Error bars refer to ± SEM of 4 – 10 replicates.
$K^+$ influx (μmol g$^{-1}$ (root FW) h$^{-1}$) vs. External $K^+$ concentration (mM)

- **NH$_4^+$-grown**
- **NO$_3^-$-grown**
Discussion

NH$_4^+$ toxicity affects many, if not most, plant species, although the mechanisms by which it occurs are still poorly understood (see review by Britto and Kronzucker, 2002). However, a common feature of NH$_4^+$ toxicity in plant systems is the suppression of tissue cation content, particularly that of potassium (Kirkby and Mengel, 1967; Kirkby, 1968; Van Beusichem et al., 1988; Engels and Marschner, 1993; Gerendás et al., 1997; Santa-María et al., 2000). K$^+$ homeostasis is also implicated as a central factor in resistance to sodium toxicity (Benlloch et al., 1994; Cuin and Shabala, 2005), and may thus play a broad role in ion stress tolerance. To better understand the role of K$^+$ in NH$_4^+$ toxicity and tolerance, we examined the influence of nitrogen source and K$^+$ supply on plant growth and K$^+$ uptake, accumulation, cytosolic pools, and root-to-shoot translocation, in ammonium-tolerant rice. We used an NH$_4^+$ concentration of 10 mM to induce toxicity under conditions that still fall within the range found in fertilized agricultural soils (Britto and Kronzucker, 2002), and the K$^+$ concentrations were chosen to represent the high- and low-affinity transport system ranges, and also to reflect soil concentrations (Reisenauer, 1966; Hawkesford and Miller, 2004). The one exception to this was the 40 mM K$^+$ treatment, which was used to test the possible limits to which elevated K$^+$ supply can relieve NH$_4^+$ stress (Britto and Kronzucker, 2002).

Rice has been traditionally considered to be an ammonium specialist (Wang et al., 1993), partly because the low oxygen environment found in rice paddy yields NH$_4^+$, rather than NO$_3^-$, as the dominant nitrogen source (Shen, 1969; Arth et al., 1998). On the other hand, it has been shown that rice seedlings are able to take up NO$_3^-$ at higher rates than NH$_4^+$ (Kronzucker et al., 2000). In support of the claim that rice may not be an NH$_4^+$ specialist under all conditions, the
present study shows that, at low concentrations of K\(^+\) (0.02 or 0.1 mM), NH\(_4^+\) nutrition suppresses growth (Table 5.1), and reduces K\(^+\) accumulation (Fig. 5.1) and influx (Fig. 5.3), relative to NO\(_3^-\) controls. Similarly, Bañuelos and coworkers (2002) found that NH\(_4^+\) suppressed K\(^+\) uptake in excised rice roots at low [K\(^+\)]\(_{ext}\). In our study, the effects observed at low [K\(^+\)]\(_{ext}\) were relieved when [K\(^+\)]\(_{ext}\) was raised to 1.5 mM and higher, indicating that NH\(_4^+\) tolerance in rice depends upon a substantial K\(^+\) supply. Nevertheless, a comparison of all growth conditions shows that the maximal biomass achieved was found not with NO\(_3^-\) but with NH\(_4^+\), and when K\(^+\) supply was moderately high (1.5 mM). This suggests that rice indeed prefers this N source as long as K\(^+\) conditions are optimized (Table 5.1).

Despite reduced growth with low [K\(^+\)]\(_{ext}\), rice seedlings were not as severely affected by NH\(_4^+\) as were seedlings of barley, considered to be an NH\(_4^+\)-sensitive species (Fig. 3.3, 3.5; Kronzucker et al. 2003b). Although growth in both species was reduced by 50% at the lowest [K\(^+\)]\(_{ext}\) (0.02 mM) with NH\(_4^+\) as the N source, the influx, cytosolic pool size, and tissue content of K\(^+\) were reduced by 80 – 90% in barley, but only by approximately 60% in rice. Moreover, increasing [K\(^+\)]\(_{ext}\) from 0.02 to 0.1 mM resulted in marked improvements in rice grown with NH\(_4^+\): growth was suppressed only by 10% and influx, [K\(^+\)]\(_{cyt}\), and tissue K\(^+\) content only by 20 – 40%, as compared with NO\(_3^-\)-grown seedlings. By contrast, barley seedlings still showed a substantial (30%) growth depression, and an even greater (60 – 90%) suppression of influx, [K\(^+\)]\(_{cyt}\), and K\(^+\) tissue content at this external [K\(^+\)]. These differences illustrate that, despite displaying some sensitivity to NH\(_4^+\), K\(^+\) homeostasis in rice shows more effective recovery from NH\(_4^+\) toxicity than barley.

Surprisingly, however, at the highest [K\(^+\)]\(_{ext}\) (40 mM), a growth decline was observed in rice seedlings, regardless of N source, even though K\(^+\) influx and tissue accumulation, cytosolic
[K\(^+\)], and \(^{42}\text{K}\(^+\) translocation were all maximized. In our previous work, a similar decline was found in NH\(_4\)\(^+\)-grown barley seedlings when [K\(^+\)]\(_{\text{ext}}\) was increased from 1.5 to 40 mM (Fig. 4.7). These reductions in growth under the extreme K\(^+\) condition may in part be a consequence of the energetic drain on root cells catalyzing substantial futile cycling of both K\(^+\) and NH\(_4\)\(^+\) under high nutrient supply (Fig. 3.7, 4.7; Britto et al., 2001, 2002; Britto and Kronzucker, 2006).

Why the steady-state influx of K\(^+\) at 40 mM should be substantially (about 40%) higher under NH\(_4\)\(^+\) nutrition than under NO\(_3\)\(^-\), is an intriguing question, particularly when both NH\(_4\)\(^+\) and K\(^+\) can have a depolarizing effect on the plasma membrane, thus reducing the driving force for K\(^+\) entry (Kronzucker et al., 2001). A stimulation of low-affinity K\(^+\) influx by NH\(_4\)\(^+\) was also seen in direct measurements of K\(^+\) influx following brief exposure (5 min) of seedlings grown at 0.1 mM [K\(^+\)]\(_{\text{ext}}\) to higher K\(^+\) concentrations (Fig. 5.6). This shows that NH\(_4\)\(^+\)-grown plants have significantly enhanced K\(^+\) influx under non-steady-state conditions, relative to NO\(_3\)\(^-\) controls. Indeed, at the highest [K\(^+\)]\(_{\text{ext}}\) tested in this experiment, the influx of K\(^+\) was more than double that of seedlings grown with NO\(_3\)\(^-\) (Fig. 5.6). Under such non-steady-state conditions as shown in Figure 5.6, NH\(_4\)\(^+\) appears to “prime” K\(^+\) influx, allowing the plant to capitalize upon a transient flush of K\(^+\) in the dynamic soil environment. Such a priming mechanism may be the result of K\(^+\) utilizing NH\(_4\)\(^+\) transporters, as has been suggested by our recent investigation in barley (see Chapter 4), which showed an immediate reduction in NH\(_4\)\(^+\) influx upon elevation of [K\(^+\)]\(_{\text{ext}}\). NH\(_4\)\(^+\) transport has been shown to follow a pattern of uptake similar to K\(^+\), with a high-affinity system at micromolar [NH\(_4\)\(^+\)]\(_{\text{ext}}\), and a low-affinity one at millimolar concentrations (Kronzucker et al., 1996), but a peculiar aspect of low-affinity NH\(_4\)\(^+\) transport is that it is not downregulated by high plant N status, but, on the contrary, is substantially increased (Wang et al., 1993; Rawat et al., 1999; Min et al., 2000; Cerezo et al., 2001). It has been suggested that this increase is due to the
induction, or enhancement, of low-affinity \( \text{NH}_4^+ \) transport by \( \text{NH}_4^+ \) itself (Cerezo et al., 2001). Therefore, it is possible that under high \( [\text{NH}_4^+]_{\text{ext}} \), \( \text{K}^+ \) utilizes an induced \( \text{NH}_4^+ \) transporter to enter the plant cell, if \( \text{K}^+ \) is present at a sufficiently high concentration, thus accounting for the increased \( \text{K}^+ \) flux. \( \text{K}^+ \) transport via \( \text{NH}_4^+ \)-specific pathways has been suggested to occur in leaves of \textit{Brassica napus} by Nielsen and Schjoerring (1998), and the existence of common pathways for the two ions is further substantiated by numerous indications that \( \text{NH}_4^+ \) influx can occur via \( \text{K}^+ \) transporters (Scherer et al., 1984; Vale et al., 1987; Wang et al., 1996; White, 1996), a phenomenon that has also been postulated for \( \text{Na}^+ \) influx (e.g., Kader and Lindberg, 2005).

It should be pointed out, however, that the effect shown in Fig. 5.6, when seedlings were transferred from a condition of 0.1 mM \( [\text{K}^+]_{\text{ext}} \) to higher \( \text{K}^+ \) concentrations, was only temporary. At the steady state, \( \text{K}^+ \) influx parity between \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) growth conditions was achieved at 1.5 mM \( [\text{K}^+]_{\text{ext}} \), signaling a longer-term downregulation of \( \text{NH}_4^+ \)-related component(s) of \( \text{K}^+ \) acquisition. The enhanced \( \text{K}^+ \) influx by \( \text{NH}_4^+ \) seen at the 40 mM steady-state condition, may also be the result of longer-term adaptations, a view supported by others who have found that \( \text{NH}_4^+ \) can enhance \( \text{K}^+ \) uptake in plant species when \( \text{K}^+ \) is supplied under nutrient-replete conditions (Daliparthy et al., 1994 and references therein).

A broad correlation was seen between unidirectional \( \text{K}^+ \) influx (Fig. 5.3) and cytosolic \([\text{K}^+]\) (Fig. 5.4) in root cells. Accordingly, a number of different set points for \([\text{K}^+]_{\text{cyt}}\) were observed as the flux increased, confirming our previous conclusion that the homeostatic control of cytosolic \( \text{K}^+ \) pools is not as rigid as generally thought (Fig. 3.6; Kronzucker et al., 2003b, 2006). A particularly striking observation was found at 1.5 mM \( [\text{K}^+]_{\text{ext}} \), in plants growing with
either N source: at this intermediate K$^+$ concentration, a dip in [K$^+$]$_{cyt}$ was seen relative to the 0.1 or 40 mM levels of [K$^+$]$_{ext}$. This pattern has been observed before for nitrate-grown barley (Fig. 3.6; Kronzucker et al., 2003b, 2006), and it receives strong confirmation in the present study by being visible in a second species, and under two nitrogen regimes. The reasons for this decline are not clear, but may be associated with the switch between a condition dominated by high-affinity K$^+$ transport to one dominated by a low-affinity system (Britto and Kronzucker, 2006).

A high correlation was found in rice between root [K$^+$]$_{cyt}$ (Fig. 5.4) and both shoot K$^+$ content (Fig. 5.7a; $R^2 = 0.82$) and $^{42}$K$^+$ transport to the shoot (Fig. 5.7b; $R^2 = 0.96$). This suggests that the cytosolic concentration of K$^+$ in the root is an important driver of long-distance K$^+$ transport. A similar conclusion was derived for barley seedlings, grown using identical (low K$^+$ and high N) nutrient conditions, with NH$_4^+$ suppressing [K$^+$]$_{cyt}$ by 70%, and shoot transport of K$^+$ by 90% (Kronzucker et al., 2003b). Root-to-shoot K$^+$ translocation is thought to be mediated (in Arabidopsis) by at least one outwardly-rectifying, Shaker-type channel, designated as SKOR (Gaymard et al., 1998; Mäser et al., 2001). Our findings suggest that NH$_4^+$ may act directly on shoot K$^+$ transporters, such as SKOR, or may disrupt K$^+$ translocation to the shoot by reducing the driving force for shoot transport (Liu et al., 2006). Such effects may be reduced in rice, unlike in barley, as rice has been shown to maintain lower [NH$_4^+$]$_{cyt}$ than found under identical conditions in barley (Britto et al., 2001). Moreover, elevating [K$^+$]$_{ext}$ may mitigate the effects of NH$_4^+$ upon K$^+$ shoot translocation in rice, by reducing [NH$_4^+$]$_{cyt}$, as was demonstrated recently in barley (Fig. 4.6). In that study, increasing [K$^+$]$_{ext}$ from a HATS-mediated to LATS-mediated transport condition, reduced [NH$_4^+$]$_{cyt}$ by three to four times.

Our hypothesis, that K$^+$ acquisition and homeostasis in rice is resistant to NH$_4^+$ nutrition, was only partially borne out. Indeed, as with most other plant species, some disruption of K$^+$
Figure 5.7 Relationship between (A) shoot $K^+$ content and the root cytosolic $K^+$ concentration and (B) shoot $^{42}K^+$ content and root cytosolic $K^+$ concentration in rice seedlings. Error bars refer to ± SEM of 5 – 18 replicates. Regression equations are: (A) $y = 1.84x + 39.5$, with $R^2 = 0.82$ and (B) $y = 5565x + 75.8$, with $R^2 = 0.96$. 
A

Cytosolic $K^+$ concentration (mM)

$K^+$ content in shoot ($\mu$mol g$^{-1}$ (shoot FW))

B

Cytosolic $K^+$ concentration (mM)

$^{42}K^+$ content in shoots (cpm g$^{-1}$ (root FW) x 10$^6$)
acquisition and distribution was seen under low K\+(reflective of high-affinity K\+ transport conditions). However, at 1.5 mM [K\+]_{ext}, growth was markedly greater under NH\(_4\)+ nutrition, and NH\(_4\)+ stimulated K\+ acquisition at elevated [K\+]_{ext}, resulting in increased K\+ transport into root cells, tissue K\+, and \(^{42}\)K\+ translocation to the shoot. Importantly, these apparent advantages translate into superior growth at the moderate LATS concentration of 1.5 mM [K\+]_{ext}. At 40 mM, by contrast, increased K\+ acquisition was associated with a growth depression, which may be attributable to the combined energy demands of futile NH\(_4\)+ and K\+ cycling at the root plasma membrane, as demonstrated elsewhere for the two nutrient ions (Fig. 3.7, 4.7; Britto et al., 2001, 2002). The efficient recovery from NH\(_4\)+ toxicity, and superior growth of rice with NH\(_4\)+, under moderate K\+ conditions, demonstrate the close association of these two ions in the context of optimal plant growth, and may offer a focal point for the bioengineering of ammonium tolerance into sensitive crop genotypes.
CHAPTER 6:

Conclusions and future perspectives
Since the pioneering research of Epstein and coworkers (1963), our understanding of plant $K^+$ transport has advanced such that we can now describe the influx mechanisms originally classified as “mechanism one” and “mechanism two” in terms of specific proteins. However, significant gaps remain in our understanding of $K^+$ transport, particularly in the low-affinity (“mechanism two”) transport range. The goal of the present work was to address this deficiency, by examining (1) the pathways mediating, (2) the driving forces affecting, and (3) the energetic consequences of $K^+$ transport.

Prior to the present studies, it was generally thought that $[K^+]_{cyt}$ was homeostatically maintained at approximately 100 mM (Leigh and Wyn Jones, 1984; Walker et al., 1996), except under $Na^+$ or $NH_4^+$ stress (Hajibagheri et al., 1987, 1988; Speer and Kaiser, 1991; Flowers and Hajibagheri, 2001; Kronzucker et al., 2003). However, LATS conditions had not been extensively examined in this context. The present work shows that as $[K^+]_{ext}$ increased, $[K^+]_{cyt}$ varied from approximately 30 to 200 mM (Fig. 3.6, 5.4).

Under LATS-mediated conditions, $K^+$ influx is known to rise dramatically with $[K^+]_{ext}$ (Gierth and Mäser, 2007), but our investigations determined that $K^+$ efflux increased to an even greater extent (Fig. 3.3). $K^+$ efflux was approximately 80% of influx under some conditions. This phenomenon of plasma-membrane futile ion cycling has also been documented under LATS-mediated conditions for $NH_4^+$ as well as a number of other ions, indicating that this may be a common characteristic for all LATS-mediated ion transport (Britto and Kronzucker, 2006). However, plasma-membrane $NH_4^+$ futile cycling is considered detrimental to plant growth (Kronzucker et al., 2001).

$K^+$ efflux under HATS-mediated conditions is considered to be a thermodynamically passive, channel-mediated flux, but less is known about $K^+$ efflux under LATS conditions. Our
investigations indicate that K\(^+\) efflux is active, against its electrochemical gradient, under high [K\(^+\)]\(_{\text{ext}}\) (Table 3.1). Moreover, the calculated energetic costs of mediating sizable active K\(^+\) fluxes (such as efflux) are potentially detrimental to the plant (Fig. 3.7, 4.7). Similarly, LATS-mediated NH\(_4^+\) fluxes have been found to carry a substantial energy burden and have been suggested to contribute to NH\(_4^+\) toxicity (Britto et al., 2001; Kronzucker et al., 2001).

High K\(^+\) influx and efflux under LATS-mediated conditions were accompanied by a shortening of the cytosolic half-time of K\(^+\) exchange, from approximately 24 min under HATS conditions, to as short as five min under LATS conditions (Fig. 2.2, 3.1, 3.2). The combination of high efflux and short cytosolic half-times magnify the loss of \(^{42}\)K\(^+\) during direct tracer influx protocols, even with short experimental time periods (Britto and Kronzucker, 2001). These results illustrate the difficulty associated with influx measured under LATS conditions, where the “face-value” estimate for influx may fall short of the real influx value (Fig. 2.4).

An interesting consequence of increasing [K\(^+\)]\(_{\text{ext}}\) from a HATS- to a LATS-mediated condition, was that the rapid, futile plasma-membrane cycling of NH\(_4^+\) was reduced (Fig. 4.2). NH\(_4^+\) influx, efflux, and [NH\(_4^+\)]\(_{\text{cyt}}\) fell significantly as [K\(^+\)]\(_{\text{ext}}\) was raised; both in the short and longer terms (Fig. 4.2, 4.3, 4.4, 4.6). When both NH\(_4^+\) and K\(^+\) futile cycling were minimized, O\(_2\) consumption by roots was also minimized, and plant mass was the largest (Fig. 4.7).

The importance of the interaction between K\(^+\) and NH\(_4^+\) was also illustrated by NH\(_4^+\) sensitivity and tolerance in rice. Under high [NH\(_4^+\)]\(_{\text{ext}}\), low [K\(^+\)]\(_{\text{ext}}\) insufficiently protected rice seedlings from NH\(_4^+\) toxicity. However, plant growth, K\(^+\) influx, and K\(^+\) tissue content were maximized when K\(^+\) and NH\(_4^+\) were both provided in their respective LATS ranges (Table 5.1; Fig. 5.1, 5.3). The observation that NH\(_4^+\) appears to prime K\(^+\) uptake suggests a synergistic interaction between these nutrients in rice (Fig. 5.6).
Futile nutrient cycling appears to be a universal ion transport phenomenon under LATS-mediated conditions (Britto and Kronzucker, 2006). However, such rapid, futile cycling results in very low cellular K\(^+\)-use efficiency, whereby sizable plasma-membrane influx is counteracted by substantial simultaneous efflux (Fig. 3.3, 4.2). It is intriguing to ask why such a mechanism exists under conditions of luxury consumption, where influx outstrips nutrient demand and storage capacity. What were the ecological driving forces that conserved this mechanism over evolutionary history? By measuring plant growth response under LATS conditions, genotypes may be found that could be used to examine the degree of futile ion cycling. Tolerance to high nutrient conditions may be a result of greater cellular ion-use efficiency.

It is interesting to consider what molecular entities are mediating the substantial K\(^+\) efflux, against its electrochemical gradient. Active K\(^+\) efflux is most likely energetically coupled to the passive influx of H\(^+\). Such a mechanism has been demonstrated for Na\(^+\) efflux, mediated by SOS1 in the plasma membrane (Shi et al., 2000). No plasma membrane equivalent to SOS1 has been found for K\(^+\).

Similarly, it is interesting to speculate about the molecular entities mediating NH\(_4\)\(^+\) influx and efflux under NH\(_4\)\(^+\) LATS conditions. The evidence presented here suggests that NH\(_4\)\(^+\) influx may be catalyzed by NH\(_4\)\(^+\) specific channels, along with NSCCs or K\(^+\) channels such as AKT1. Similar to K\(^+\) efflux, NH\(_4\)\(^+\) efflux is against its electrochemical gradient and likely is energetically coupled to the passive influx of H\(^+\).

An important consequence of the estimated oxygen consumption necessary to drive K\(^+\) or NH\(_4\)\(^+\) transport (Fig. 4.7) is that current models describing the energetics of ion transport may be inadequate. Under conditions of high nutrient transport, cation and anion cotransport mechanisms may be functioning in plants, in addition to known transporters that have been
characterized to date, which utilize ATP or the proton gradient in energetically-inefficient ways. Suggestions of such flux-coupling mechanisms are prevalent throughout the animal kingdom (Russell, 2000), in the form of Na\(^+\)-Cl\(^-\), K\(^+\)-Cl\(^-\), or Na\(^+\), K\(^+\), Cl\(^-\) transporters. Such transporters are virtually unknown in plants, with the exception of a cation-chloride channel recently identified in *A. thaliana*, which mediates Na\(^+\), K\(^+\), and Cl\(^-\) fluxes (Colmenero-Flores et al., 2007). Cotransport mechanisms have also been postulated based upon observations of anions altering the influx of cations (Epstein et al., 1963; Rains and Epstein, 1967a; Kochian et al., 1985). It has been shown that LATS-mediated K\(^+\) or Na\(^+\) influx can be accelerated with Cl\(^-\) provision, relative to other anions, such as SO\(_4^{2-}\) or PO\(_4^{3-}\) (Epstein et al., 1963; Rains and Epstein, 1967a; Kochian et al., 1985). Moreover, inhibition of the Cl\(^-\) flux can reduce K\(^+\) transport (Kochian et al., 1985). Future investigations of ion cotransport are necessary, as they may lead to the discovery of novel energy-utilization mechanisms in plants.

As discussed in Chapter Two, influx is the parameter most researchers are interested in determining, as it is the primary step of mineral acquisition. However, studies quantifying nutrient uptake, particularly in LATS conditions, must consider: the amount of physical disturbance plants are subjected to during an experiment; possible accelerated cellular half-times of exchange; and, greater rates of efflux with increasing nutrient provision. It is clear that channel-mediated fluxes can be difficult to measure accurately, but these considerations ensure influx is not underestimated. The recommendations presented should also be applied to examinations of Na\(^+\) or heavy-metal influx, where it has been found that ion influx is likely channel-mediated (Arazi et al., 1999; Davenport and Tester, 2000; Sunkar et al., 2000; Amtmann et al., 2001; Maathuis and Sanders, 2001; Paganutto et al., 2001; Demidchik and Tester, 2002; Essah et al., 2003; Gobert et al., 2006; Wang et al., 2007; Wojas et al., 2007).
The synergistic effect between K\(^+\) and NH\(_4\)^+ in rice demands further examination. It is unclear how NH\(_4\)^+ primed K\(^+\) influx, under K\(^+\) HATS-mediated conditions, or how NH\(_4\)^+ stimulated growth and tissue K\(^+\) accumulation under K\(^+\) LATS-mediated conditions. The implications of finding optimal K\(^+\):NH\(_4\)^+ ratios for growth may be significant if such work could be applied to agricultural settings.

The preceding work contributes new and fundamental discoveries to our understanding of K\(^+\) transport. While some questions remain unanswered, and new interesting observations demand explanation, the primary findings of: 1) rapid, futile cycling of K\(^+\) under LATS conditions; 2) a dynamic cytosolic K\(^+\) concentration; 3) active K\(^+\) efflux under LATS conditions; 4) rapid, K\(^+\)-mediated, reduction of NH\(_4\)^+ futile cycling; 5) NH\(_4\)^+ priming of K\(^+\) uptake in rice; 6) the energetic burden of futile cycling of K\(^+\) and NH\(_4\)^+; and, 7) a resolution of the difficulties associated with measuring ion influx under LATS conditions, are exciting additions to our understanding of K\(^+\) transport.


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APPENDIX I:

Technical note on compartmental analysis using tracer efflux

(CATE)
Examination of ion transport processes and subcellular compartmentation can yield valuable insights into cell physiology, but their accurate determination presents many unique challenges. The flux value most often sought by ion transport researchers is that of influx, although many researchers settle for a net approximation of ion uptake. Using K\(^+\) as an example, net flux can be determined by a number of means, the easiest being a depletion experiment, in which roots of intact plants (or excised plant tissue or protoplasts) are placed in a solution of known K\(^+\) concentration for a period of time. Subsequently, the plant is removed, and the [K\(^+\)] in solution is determined by flame or atomic absorption spectroscopy, or using a K\(^+\)-selective electrode (Bañuelos et al., 2002). Extracellular K\(^+\)-selective microelectrodes can also be used to directly determine a net flux using intact or excised plant tissue (Cuin and Shabala, 2000). The radiometric technique of direct influx has the advantage of directly monitoring the amount of K\(^+\) (using a radioactive tracer) that accumulates over a period of time in the plant tissue (Epstein et al., 1963; Glass, 1976; Kochian and Lucas, 1982; Santa-Maria et al., 2000). This technique involves labelling the plant tissue with a radioactive tracer, preferably a K\(^+\) isotope such as \(^{42}\)K\(^+\) (although \(^{86}\)Rb\(^+\) is more often used), for a short period of time (often ranging between five and 30 minutes). Following the labelling period, the plant is removed from the radioactive solution and washed to remove radioactivity bound to the surface of the plant tissue (surface film), or adhering to the fixed charges of the cell wall (Donnan free space). Over short periods of time and under conditions of low K\(^+\) efflux, this technique can accurately estimate K\(^+\) influx; however, as labelling times increase, or if efflux is a significant proportion of influx, or if the cytosolic pool is rapidly exchanging, this technique can drastically underestimate the influx value (Britto and Kronzucker, 2001, 2006). While depletion, electrode, and direct tracer uptake measurements
provide useful information concerning \( \text{K}^+ \) transport, they only assist in determining a single parameter (influx or net flux) of \( \text{K}^+ \) transport.

Throughout my thesis I have employed the technique of compartmental analysis by tracer efflux. CATE experimentation involves the capture of radioactive tracer released by the plant in discrete units, following a prolonged labelling of the plant with radioactive tracer (e.g., \( ^{42}\text{K} \) or \( ^{13}\text{N} \)). CATE simultaneously provides information not only about influx, but also about multiple other fluxes (efflux, net flux, and flux to the xylem), as well as kinetic constants of exchange, and subcellular compartmentation, of the traced ion. CATE has been used extensively to investigate a wide range of plant processes, including: ion fluxes and compartmentation across membranes for \( \text{K}^+ \), \( \text{NH}_4^+ \), \( \text{Na}^+ \), \( \text{NO}_3^- \), and \( \text{Cl}^- \) (Cram, 1968; Pallaghy and Scott, 1969; Macklon and Higinbotham, 1970; Pierce and Higinbotham, 1970; Pitman, 1971; Macklon, 1975; Behl and Jeschke, 1982; Jeschke, 1982; Mills et al., 1985; Lee and Clarkson, 1986; Siddiqi et al., 1991; Kronzucker et al., 1995a, b, c, d; Min et al., 1999; Britto et al., 2001, 2002, 2004; Kronzucker et al., 2003b; Ritchie, 2006); salinity stress (Hajibagheri et al., 1988); stomatal function (MacRobbie, 1981, 1995); forest succession (Kronzucker et al., 1997, 2003a); metal tolerance (Lasat et al., 1998, 2000; Zhu et al., 2000; Pedas et al., 2005); synergistic effects of mineral nutrition (Kronzucker et al., 1999); action of hormones (Hellwege and Hartung, 1997; Jovanovic et al., 2000); and compartmentation of herbicides (DiTomaso et al., 1993; Lasat et al., 1997).

CATE, as will be described here, will be limited to my use with \( ^{42}\text{K}^+ \) and intact plants, and to the main parameters determined by CATE; however, this description can be generalized to include other tracers, with only the method of isotopic quantification changing to suit the tracer used (e.g. mass spectrometry for studies involving nitrogen-15). The primary assumptions that govern CATE experimentation are: 1) that there are no kinetic limitations within a
compartment; i.e., that each compartment behaves as a well-stirred medium (Poole, 1971; Zierler, 1981); and, 2) that the system under examination is at steady state. Under steady-state conditions, fluxes into or out of compartments are constant and solute pools are not changing; thus, rates of change within a compartment at steady state are governed by first-order kinetics (Pallaghy and Scott, 1969; MacRobbie 1971, 1981; Poole, 1971; Walker and Pitman, 1976; Zierler, 1981; Britto and Kronzucker, 2001a, 2003a, b, c). In order to satisfy the steady-state assumption our plants are grown in, and experiments are conducted in, walk-in growth rooms that maintain daytime and nighttime temperatures, and light conditions. In addition, the hydroponic nutrient solutions that our plants are grown in are exchanged frequently, ensuring nutrient conditions are constant during plant growth and experimentation. Finally, in all experiments, \(^{42}\text{K}^+\) is used rather than \(^{86}\text{Rb}^+\). Several studies have shown that plants can discriminate between the alkali cations \(^{1+}\text{Rb}^+\) and \(^{1+}\text{K}^+\), with \(^{1+}\text{Rb}^+\) even inhibiting \(^{1+}\text{K}^+\) uptake. These effects have been found across a broad range of external \(^{1+}\text{K}^+\) conditions and in a variety of plant systems (Cline and Hungate, 1960; Schaedle and Jacobson, 1967; West and Pitman, 1967; Maas and Leggett, 1968; Marschner and Schimansky, 1968; Jacoby, 1975; Jacoby and Nissen, 1977; Rhoads et al., 1977; Reed and Collins, 1981; Behl and Jeschke, 1982; Schachtman and Schroeder, 1994). Over extended experimental time periods, use of \(^{1+}\text{Rb}^+\) may affect cytosolic enzyme activity (Evans and Sorger, 1966; Nitsos and Evans, 1969; Sueltel, 1970; Page and Di Cera, 2006), and even lead to toxic symptoms as a result of the cellular accumulation of \(^{1+}\text{Rb}^+\) (El-Sheikh and Ulrich, 1970).

CATE commences by labelling a group of plant roots with \(^{42}\text{K}^+\) in a solution identical to the growth solution, except for the presence of the radioactive tracer. Groups of three to five plants were commonly used, however, groups as large as 16 were examined and found not to
produce different results as compared to smaller groups, indicating that diffusion limitation was negligible. Following the labelling period (approximately 1 h, to avoid significant labelling of subcellular compartments other than the cytosol - e.g. the vacuole, Memon et al., 1985a), the plants are removed from the radioactive labelling solution and bathed in successive aliquots of nonradioactive solutions, identical to the growth solution, using a timed series (Fig. AI.1). The radioactive aliquots are counted using a gamma counter, corrected for isotopic decay and plant mass, and used to generate a semilogarithmic plot of the rate of tracer release versus time.

Following first-order kinetics, the rate of tracer release is described by the equation:

\[ \varphi_t^* = \varphi_o^* e^{-kt} \]

Where \( \varphi_t^* \) represents the radioactive efflux at time \( t \), \( \varphi_o^* \) represents the maximal radioactive efflux at time zero, and \( k \) represents the kinetic rate constant of exchange for the compartment (the half-time of exchange is determined by the calculation \((\ln 2)/k\)). A semilogarithmic plot of this equation gives a straight line, with the equation becoming:

\[ \ln \varphi_t^* = \ln \varphi_o^* - kt \]

The compoundly exponential plot shown in Figure AI.1 has three overlapping phases of efflux, each representing \( ^{42}\text{K}^+ \) release from a different compartment (surface film, Donnan free space, or cytosol), and graphically represented by the straight lines. The three compartments are kinetically distinct, each releasing \( ^{42}\text{K}^+ \) at a different rate, with the fastest being the surface film (with a half-time of exchange of approximately 6 s) and the slowest being the cytosol (with a half-time of exchange of approximately 30 min, under moderate \( \text{K}^+ \) supply).

Beginning at the tail end of the plot (\( t = 30 \) min, Fig. AI.1), where the radioactive-release contribution of the prior two compartments will be negligible, linear regression is used to approximate a function (\( \ln \varphi_t^* = \ln \varphi_o^* - kt \)) representing the \( ^{42}\text{K}^+ \) efflux from the cytosol (where
Figure A1.1 Representative $^{42}\text{K}^+$ efflux pattern in roots of barley seedlings grown at 0.1 mM $\text{K}^+$ and 10 mM $\text{NH}_4^+$. Regression lines shown representing the surface film (red), Donnan free space (blue), and cytosol (black).
\[ \text{Ln} \left( ^{42}\text{K}^+ \text{ efflux (cpm released g}^{-1}\text{ (root FW) h}^{-1}\text{)} \right) \]

- **Surface film**
  - \( k = 12.53 \text{ min}^{-1} \)
  - \( t_{1/2} = 0.10 \text{ min} \)

- **Donnan free space**
  - \( k = 0.75 \text{ min}^{-1} \)
  - \( t_{1/2} = 0.92 \text{ min} \)

- **Cytosol**
  - \( k = 0.02 \text{ min}^{-1} \)
  - \( t_{1/2} = 29.7 \text{ min} \)
\( \varphi_{co}^* \) represents the maximal radioactive efflux from the cytosol at time zero, and corresponding to the antilog of the y-intercept). Analysis of the Donnan free space is carried out in a similar manner, but the contribution of the cytosol to \(^{42}\text{K}^+\) efflux in this region of the graph must first be subtracted before conducting linear regression for the Donnan phase (and similarly the Donnan-phase contribution to tracer efflux must be subtracted before analysing the surface film).

The chemical (nonradioactive) efflux of \( \text{K}^+ \) from the cytosol is determined by dividing \( \varphi_{co}^* \) by the specific activity of \(^{42}\text{K}^+\) in the cytosol \( (SA_{cyt}) \) at elution time zero, determined using the equation:

\[
SA_{cyt} = SA_o \cdot (1 - e^{-kt})
\]

Where \( SA_o \) is the specific activity of the labelling solution and \( t \) is labelling time. The net flux is determined by taking the radioactive counts remaining in the plant sample after the experiment, subtracting the amount of residual tracer that would be removed should experimentation continue (i.e. until infinity, \( \infty \)), and then dividing by \( SA_o \), root weight, and labelling time. The subtracted residual tracer is determined by integrating the function representing the rate of tracer efflux, between the end of experimentation, \( t_{end} \), and \( \infty \):

\[
\int_{t_{end}}^{\infty} \varphi_{co}^* e^{-kt} dt = \left[ -\frac{\varphi_{co}^*}{k} e^{-kt} \right]_{t_{end}}^{\infty}
\]

Influx is simply the sum of the net flux and efflux.

The cytosolic concentration is calculated first by quantifying the tracer lost from this compartment. This can be accomplished by integrating the function representing the rate of tracer efflux, between \( t = 0 \) and \( t = \infty \):

\[
\int_{0}^{\infty} \varphi_{co}^* e^{-kt} dt = \left[ -\frac{\varphi_{co}^*}{k} e^{-kt} \right]_{0}^{\infty} = \frac{\varphi_{co}^*}{k}
\]
The total radioactive content of the cytosol prior to elution ($Q^*$) is equal to the quotient of the amount of radioactive tracer efflux (above value), divided by the ratio of radioactive tracer efflux to all other removal fluxes from the cytosol (which is equivalent to radioactive efflux divided by radioactive influx):

$$Q^* = \frac{\phi_{oc}^*}{k} = \left\{ \frac{\phi_{co}^*}{\phi_{oc}^*} \right\}$$

The cytosolic concentration is found by dividing the term above by $SA_{cyt}$ and correcting for the cytosolic volume (approximately 5% of the cell volume, see Table 1.1 for estimates). Simplified, this calculation appears as: $Q = t_{1/2} \phi_{oc} \Omega$, where $Q$ is the cytosolic concentration, $t_{1/2}$ is the half-time of cytosolic exchange, $\phi_{oc}$ is the influx, $\Omega$ and is a proportionality constant correcting for cytosolic volume.

Prior to engaging in CATE analysis, rigorous phase testing must take place, in order to appropriately assign compartment identity (Pitman and Saddler, 1967; Pallaghy and Scott, 1969; Pierce and Higinbotham, 1970; Macklon et al., 1975; Memon et al., 1985a; Mills et al., 1985; Kronzucker et al., 1995a, b, c, d; Britto et al., 2001; Kronzucker et al., 2003b). While each compartment has a unique half-time of exchange, various experimental conditions can adjust these values, reducing the difference between compartments. In such cases, linear regression alone may not be suitable for identifying individual compartments (Cheeseman, 1986). Other means of compartment identification can include the use of metabolic inhibitors, treatment with detergents or high temperature to disrupt membranes, or the use of competing ions or transport inhibitors, with the expectation that compartments will respond differently to such treatments, providing additional evidence for compartment identification (Kronzucker et al., 1995d; Britto
and Kronzucker, 2003a). For instance, the cytosolic compartment can be targeted by treating a plant with sodium dodecyl sulphate, a detergent that will disrupt the plasma membrane of cells, along with the radioactive efflux from the cytosol. However, such a treatment will have little to no effect on the radioactive efflux from the surface film or Donnan free space. Inappropriate phase identification has led to confusion within the literature concerning the size of ion fluxes or compartments, or the kinetics of exchange within a phase (Macklon, 1975; Macklon and Sim, 1981; Ritchie, 2006).

Interestingly, while CATE experiments have been criticized more than direct influx experiments, plants are subjected to greater stress and perturbation during direct influx experiments, as often short time periods (e.g. five minutes) are used, coupled with multiple bathing solutions, for the loading and removal of tracer, all of which are factors contributing to ion flux disruption (Gronewald et al., 1979; Gronewald and Hanson, 1980; Chastain and Hanson, 1982; Rincon and Hanson, 1986; Bloom and Sukrapanna, 1990; Hommels et al., 1990; Aslam et al., 1996; ter Steege et al., 1998). Direct influx experiments are also complicated by concurrent efflux that can substantially reduce tracer accumulation, resulting in the dramatic misinterpretations of influx data, while CATE experiments are more comprehensive (Cram, 1969; Cram and Laties, 1971; Presland and McNaughton, 1984; Lee and Drew, 1986; Britto and Kronzucker, 2001a). Concern over the use of CATE has also been raised on the grounds that the technique offers not a cell-specific estimate, but an average of fluxes or compartment concentrations, because the plant organs examined contain a variety of cell types. However, this is no different than the findings provided by enzyme kinetic studies, NMR signals, or mRNA detection (Britto and Kronzucker, 2003a). Moreover, the integrated flux analysis of CATE provides a whole-tissue or whole-plant understanding of cellular mechanisms.
In addition to CATE, the following studies also relied heavily upon a subsampling-based procedure (SCATE) described elsewhere (Britto et al., 2006). Although the details of analysis are slightly different for a SCATE protocol, the assumptions that were discussed for CATE also apply to SCATE.
APPENDIX II:

\[ K^+ \text{ fluxes under non-steady-state conditions} \]
The kinetics of $\text{K}^+$ influx have been described by measuring the response of influx to shifts in $[\text{K}^+]_{\text{ext}}$ (Fig. 1.1, Epstein et al., 1963; Glass, 1976, 1978; Cheeseman and Hanson, 1979; Kochian and Lucas, 1982, 1983; Wrona and Epstein, 1985; Maathuis and Sanders, 1996b; Santa-María et al., 1997, 2000; Rubio et al., 2000). However, while the potentially large effect of concurrent efflux on $\text{K}^+$-influx measurements have been demonstrated here (Chapter 2) and elsewhere (Cram, 1969; Britto and Kronzucker, 2001) this parameter is universally neglected during such studies. Under steady-state nutritional provision, $\text{K}^+$ efflux has been shown to increase substantially with increasing $[\text{K}^+]_{\text{ext}}$ (Fig. 3.1, 3.2), but the contribution of efflux under non-steady-state ($\text{K}^+$-shift) conditions has not been investigated until now. Here, using the radiotracer $^{42}\text{K}^+$ with compartmental analysis (CATE and SCATE) and DI protocols in intact barley seedlings, we examine the magnitude of $\text{K}^+$ efflux following a shift in $[\text{K}^+]_{\text{ext}}$.

We found that $\text{K}^+$ influx increased immediately with increasing $[\text{K}^+]_{\text{ext}}$ (Fig. AII.1), confirming other studies (Epstein et al., 1963; Glass, 1976, 1978; Cheeseman and Hanson, 1979; Kochian and Lucas, 1982, 1983; Wrona and Epstein, 1985; Maathuis and Sanders, 1996b; Santa-María et al., 1997, 2000; Rubio et al., 2000). However, under conditions of low $[\text{K}^+]_{\text{ext}}$ (0.1 mM), $^{42}\text{K}^+$ efflux was reduced when $[\text{K}^+]_{\text{ext}}$ was increased to 40 mM, within two minutes of the concentration shift (Fig. AII.2a). However, a concentration shift in the opposite direction (from 40 to 0.1 mM) did not affect the rate of $^{42}\text{K}^+$ release; this parameter remained very high (Fig. AII.2b). These results, obtained using CATE, were independently confirmed by SCATE methodology (data not shown).

The reduction of $^{42}\text{K}^+$ efflux upon an increase in $[\text{K}^+]_{\text{ext}}$ in plants grown under HATS-mediated influx conditions, is hypothesized to have occurred not because non-radioactive $\text{K}^+$ efflux decreases, but because the specific activity of $^{42}\text{K}^+$ in the cytosol was diluted. This can be
Figure AII.1 K⁺ influx isotherms for barley seedlings grown at 0.1 or 40 mM [K⁺]. Each datum is the mean ± SEM of 2 – 4 replicates. Influx was determined as outlined in Chapter 5, except the tracer loading time was 10 min for these experiments.
External \([K^+]\) (mM)

K⁺ influx (µmol g⁻¹ (root FW) h⁻¹)

- ○ 0.1 mM \([K^+]_{\text{ext}}\)
- ● 40 mM \([K^+]_{\text{ext}}\)
Figure AII.2 Effect of $[K^+]_{ext}$ shift on $^{42}K^+$ efflux from the roots of intact barley seedlings grown with 0.1 mM (A) or 40 mM (B) $[K^+]_{ext}$. The arrow indicates time of shift in $[K^+]_{ext}$ from 0.1 to 40 mM (A) or 40 to 0.1 mM (B). Each point is the mean of 4 – 14 replicates.
A

Concentration shift from 0.1 to 40 mM [K$^{+}$]$_{ext}$

B

Concentration shift from 40 to 0.1 mM [K$^{+}$]$_{ext}$
explained as an effect of the substantial increase in \( K^+ \) influx following the \([K^+]_{\text{ext}}\)-shift from 0.1 to 40 mM (Fig. AII.1), that reduces the ratio of \(^{42}K^+\) to non-radioactive \( K^+ \) in the cytosol. Consequently, less \(^{42}K^+\) was captured during the CATE protocol.

The lack of response of \(^{42}K^+\) efflux in the oppositely directed concentration shift (40 to 0.1 mM), by contrast, may indicate the maintenance of the cytosolic specific activity. In this case, \( K^+ \) influx was reduced (Fig. AII.1), and thus would not have diluted the specific activity. However, the maintenance of the very high efflux requires explanation: we hypothesize that \( K^+ \) stored in root cell vacuoles or in the shoot could be released into the cytosol, maintaining the ratio of \(^{42}K^+\) to non-radioactive \( K^+ \). Accordingly, no difference in \(^{42}K^+\) efflux would be observed.

In order to test these models of subcellular \( K^+ \) efflux, whole-plant \( K^+ \) tissue content was examined. Increasing \([K^+]_{\text{ext}}\) (from 0.1 to 40 mM), resulted in greater \( K^+ \) tissue content in both roots and shoots (Fig. AII.3a); however, reducing \([K^+]_{\text{ext}}\) from 40 to 0.1 mM caused a significant reduction in shoot \( K^+ \) content, while root \( K^+ \) content was unaffected (Fig. AII.3b). The reduction in \( K^+ \) shoot content indicated a net loss of \( K^+ \) by the plant following the \([K^+]_{\text{ext}}\)-shift. Moreover, the amount of \( K^+ \) lost from the whole plant within 1 h of the downward \([K^+]_{\text{ext}}\)-shift exactly matched \( K^+ \) efflux values previously determined using CATE (Fig. 3.1, 3.3). These observations support the two hypotheses suggesting that non-radioactive \( K^+ \) efflux is recalcitrant.

These findings are of particular importance for \([K^+]_{\text{ext}}\)-shift experiments that focus on plants grown under LATS-mediated conditions, because of the very high efflux observed both under steady-state and concentration-shift conditions. Influx measurements under these conditions will be underestimated due to the large (relative to HATS-mediated growth conditions), persistent efflux carried over from the original growth condition. This may also
**Figure AII.3** Tissue K\(^{+}\) content in roots and shoots of barley seedlings following a \([K^{+}]_{\text{ext}}\) shift from 0.1 to 40 mM (A) or 40 to 0.1 mM (B). Error bars refer to ± SEM of 6 – 21 replicates. Asterisks denote significant differences within a tissue (root or shoot) from the original, steady-state content \((P < 0.05)\).
Figure A: K⁺ content (μmol g⁻¹ (root FW)) in roots and shoots after switching to 40 mM K⁺.

Figure B: K⁺ content (μmol g⁻¹ (root FW)) in roots and shoots after switching to 0.1 mM K⁺.
affect the interpretation that K\(^+\) influx in plants grown under high [K\(^+\)\(_{\text{ext}}\)] conditions is drastically reduced relative to plants grown under low [K\(^+\)\(_{\text{ext}}\)] conditions (Kochian and Lucas, 1982).

The persistent K\(^+\) efflux following [K\(^-\)\(_{\text{ext}}\)-shift is a fascinating new contribution to the understanding of cellular flux dynamics. It is unclear why K\(^+\) influx responds immediately to [K\(^+\)\(_{\text{ext}}\)-shift, and why K\(^+\) efflux does not. Moreover, how is K\(^+\) efflux maintained at a high rate, despite a switch in the electrochemical gradient for K\(^+\) efflux?

The demonstration that shoot K\(^+\) content was reduced while root K\(^+\) content remained unchanged, in the downward concentration shift, also deserves further investigation. This observation may indicate a prioritizing of K\(^+\) reservoirs within the plant, and may provide new insights into whole-plant K\(^+\) homeostasis. It is clear that additional examinations of the mechanisms mediating K\(^+\) efflux will be necessary in order to understand the complex coordination between K\(^+\) influx, efflux, and tissue content.