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How high do ion fluxes go? A re-evaluation of the two-mechanism model of K^+ transport in plant roots

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ABSTRACT

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

1. Introduction

The two-mechanism model of potassium (K^+) acquisition in roots of higher plants is a standard of transport physiology and molecular biology [1–5]. In this model (Fig. 1A), the high-affinity transport system (HATS) predominately functions at external K^+ concentrations ($[K^+]_{ext}$) below 1 mM, and is mediated by secondarily active K^+/H^+ symporters of the HAK/KUP/KT family, including AtHAK5 in Arabidopsis (*Arabidopsis thaliana*; [4]) and HvHAK1 in barley (*Hordeum vulgare*; [6]). By contrast, the low-affinity transport system (LATS), which functions at $[K^+]_{ext}$ above 1 mM, is less well understood, although it is widely accepted to be a thermodynamically passive process mediated by K^+-selective (Shaker-like) channels (*e.g.* AtAKT1 and HvAKT1 in Arabidopsis and barley, respectively; [3–5,7]). Studies using heterologous expression systems and knock-out mutants in Arabidopsis have furthermore shown that AtAKT1 can also operate under some high-affinity conditions [8–11]. Moreover, recent investigations in the Arabidopsis double-knock-out mutant, *athak5 atakt1*, have shown that there is an additional back-up system ("BUS") that can contribute to K^+ uptake in the LATS range [12–14]. Although genetically uncharacterized, it has been suggested that BUS operates via non-selective cation channels (NSCCs), which may be gated by cyclic nucleotides [13].

One commonly observed feature of the K^+ LATS is its linearly rising kinetic response to increasing $[K^+]_{ext}$, which contrasts sharply with the saturating pattern typical of the HATS [15–19] (see also Fig. 1). From the outset, it is worth noting that this model is based solely on unidirectional fluxes measured using radiotracer methodology, and although many aspects have been corroborated by other experimental methods, the linearity of the low-affinity flux is one aspect that has not. Any attempt to investigate this important concept must therefore also employ radiotracer techniques. Moreover, although many studies have reported on a linear LATS, it is important to note that some studies have reported low-affinity K^+ influxes that saturate [1,20,21]. It is interesting to note that such studies often employ longer absorption and desorption times (*e.g.* 10-30 min each), compared to many studies that report linear fluxes, which may result in closer approximations to net K^+ fluxes, rather than unidirectional uptake (see below).

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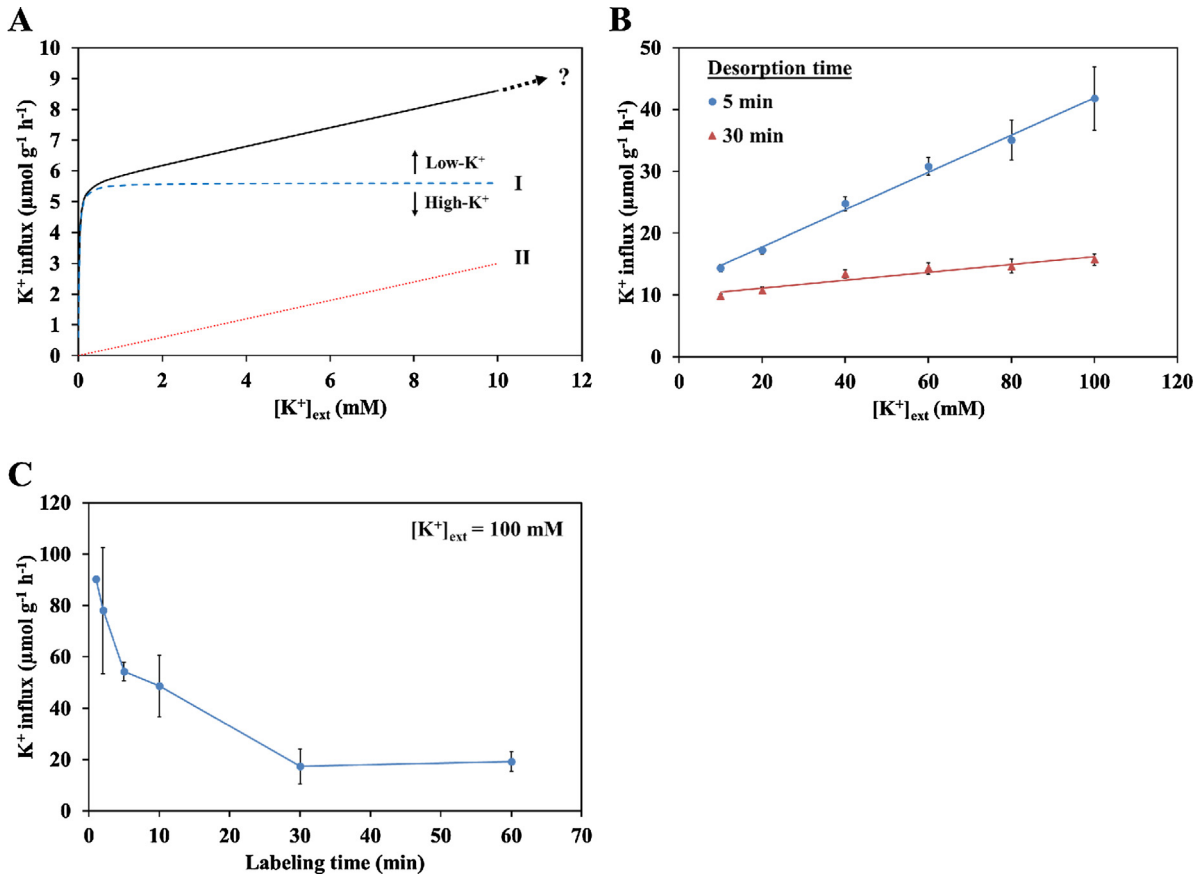

Fig. 1. The two-mechanism model of K^+ acquisition in roots of higher plants and its extension into the saline range. (A) The standing model of K^+ transport. Influx (solid black line), as a function of external $[K^+]$ ($[K^+]_{ext}$), is described as the sum of activities of two distinct transport systems: (I) the saturable high-affinity transport system (HATS; blue dashed line), which follows Michaelis–Menten kinetics and is regulated by internal K^+ status; and (II) the low-affinity transport system (LATS; red dotted line), which is linear. The HATS is primarily governed by HAK/KUP/KT transporters, whereas the LATS is governed by AKT1-channel complexes and unknown back-up system(s). Here we ask whether influx will continue to rise linearly beyond nutritionally relevant $[K^+]_{ext}$ (*i.e.* > 10 mM, and into the saline range). Redrawn from [3,63]. (B) K^+ influx into roots of intact barley seedlings, as a function of $[K^+]_{ext}$, and its dependence on desorption time (5 min, blue circles; 30 min, red triangles). Seedlings were grown on full-nutrient media supplemented with 0.1 mM K^+. Labeling time = 5 min. (C) Influx as a function of labeling time, in barley seedlings grown and measured under 100 mM K^+. Desorption time = 5 min. Throughout, error bars represent ± SEM ($n \geq 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, a linear LATS is not unique to K^+, but has been observed with many ions, including sodium (Na^+), ammonium (NH_4^+), chloride (Cl^-), and nitrate (NO_3^-) [18,22–25], as well as with organic solutes such as amino acids and sugars [26–28]. Such fluxes are generally considered to be channel-mediated, and typically show no signs of saturation at external substrate concentrations between 10 and 50 mM. Only a few studies have examined fluxes beyond this range because such conditions are often considered physiologically or ecologically irrelevant, and, moreover, potentially damaging to cells. An important exception is that of Na^+ fluxes in the context of salinity stress, where the measurement of fluxes at very high substrate concentrations (*e.g.* >100 mM) is both appropriate and routine. In this case, extraordinarily high fluxes are frequently reported, and although believed to be mediated by NSCCs, their mechanistic underpinnings remain poorly defined [29]. By contrast, much more is known about K^+ transport into roots and within the plant [5,30,31]; thus, we are better equipped to investigate the nature of the K^+ LATS, and use results from this model system to gain insight into Na^+ transport at high external concentrations.

Another critical aspect of low-affinity transport in general involves the efflux of ions, which occurs simultaneously with influx, and appears to rise more steeply than influx does, as external concentrations increase [32]. Such patterns have been observed for all major nutrient ions, including K^+, Na^+, Cl^-, NO_3^-, NH_4^+, and sulfate (SO_4^{2-}), and efflux in the low-affinity range frequently achieves rates approximating those of influx (*i.e.* efflux:influx ratios become close to unity). This futile cycling of ions typically carries with it a potentially substantial energetic burden on root systems, given that efflux or influx components of the cycle may occur against significant thermodynamic gradients [25,32,33].

High rates of efflux make the accurate measurement of low-affinity unidirectional influx, by use of tracer methods, a challenging prospect [34]. In order to limit the effect of efflux occurring during tracer-influx measurement protocols, many researchers advocate the use of very short labeling times (*e.g.* 2–5 min; [25,35,36]). For the same reason, short desorption times (*i.e.* "washes" in non-labeled solutions following uptake, to remove extracellular tracer; see Section 2.2) are often prescribed. Although such protocols have become standard in the measurement of low-affinity fluxes (*e.g.* see [29]), the assumptions that they minimize tracer efflux across the plasma membrane, and clear the roots of extracellular tracer, are rarely tested, and indeed may be untenable in some cases. For example, we recently showed that K^+ efflux across the plasma membrane in roots of intact barley seedlings ceases above a $[K^+]_{ext}$ of 1 mM (*i.e.* in the low-affinity range), and concluded that tracer release from pre-labeled roots was extracellular (apoplastic) in origin [37]. The implications from this study for low-affinity influx measurements require further exploration.

Here, we ask three fundamental questions regarding the nature of low-affinity K^+ influx in roots of higher plants: (1) Does influx

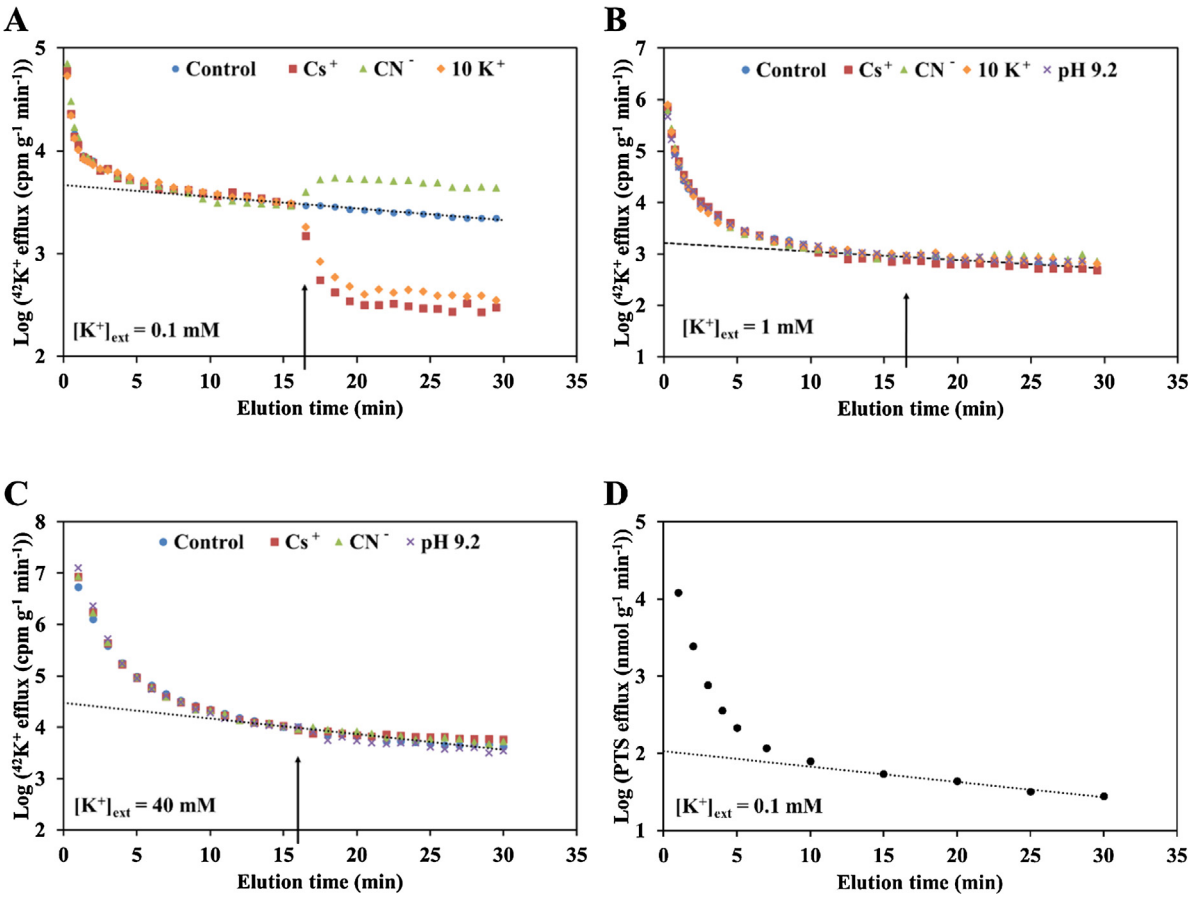

Fig. 2. Cellular K^+ efflux ceases in the low-affinity range and what is measured is extracellular (apoplastic) in origin. (A) $^{42}K^+$ efflux from roots of intact barley seedlings grown and measured at 0.1 mM K^+, and the response to the sudden (at t = 16 min; see arrow) exposure of either 10 mM Cs^+ (red squares), 1 mM CN^- (green triangles), or high (10 mM) K^+ (orange diamonds). (B and C) As in panel A, but with plants grown and measured at 1 and 40 mM K^+, respectively, and exposed to alkalinity (pH 9.2; purple crosses). (D) PTS release from pre-labeled (0.1% PTS (w/v)) roots of intact barley seedlings grown as in panel A . Under all conditions, compartmental analysis of the slowly-exchanging phase (dotted line) was conducted (see Table 1 for extracted parameters). Each point represents the mean of a minimum 3 replicates (SEM < 15% of the mean). (A and B) Redrawn from [37]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have an upper limit and eventually saturate with rising $[K^+]_{ext}$, or continue to rise indefinitely? (2) What mechanism(s) underlie its linear concentration dependence? (3) Can a better understanding of low-affinity K^+ transport help resolve the mechanisms of Na^+ fluxes under salt stress? We shall argue that transmembrane fluxes do not rise indefinitely, but saturate at relatively modest rates (*i.e.* 7–15 μmol g^{-1} root fresh weight (FW) h^{-1}), well into the saline range (≤100 mM $[K^+]_{ext}$), while the linearly rising component of the measured flux is dominated by the apoplastic movement of K^+. We believe that our findings call for a fundamental revision of flux models, and apply to low-affinity fluxes of other ions, particularly those of Na^+.

2. Materials and methods

2.1. Plant culture

Barley seeds (*H. vulgare* L., cv. 'Metcalfe') were surfaced-sterilized for 10 min in 1% sodium hypochlorite, and germinated under acid-washed sand for 3 d prior to placement in 14-L hydroponic vessels containing aerated modified Johnson's solution (0.5 mM $Ca(NO_3)_2$, 0.5 mM NaH_2PO_4, 0.25 mM $MgSO_4$, 25 μM H_3BO_3, 20 μM Fe-EDTA, 6.25 μM $CaCl_2$, 2 μM $ZnSO_4$, 0.5 μM $MnSO_4$, 0.5 μM $CuSO_4$, and 0.125 μM Na_2MoO_4), pH 6.3–6.5 (adjusted with NaOH), for an additional 4 d. Growth media were modified to provide potassium (as K_2SO_4) at 0.1, 1, 40, or 100 mM. To ensure plants remained at a nutritional steady state, solutions were exchanged on days 5 (for all K^+ levels) and 6 (for 0.1 mM K^+ plants), and experimented with on day 7. Plants were grown in a walk-in growth chamber under fluorescent lights with an irradiation of ~200 μmol photons m^{-2} s^{-1} at plant height for 16 h d^{-1}. Daytime temperature was 20 °C, night-time temperature was 15 °C, and relative humidity was ~70%.

Seeds of Arabidopsis (*A. thaliana* L.) t-DNA insertion line *athak5 atakt1* (Col-0 background; [13]) were surfaced sterilized for 5 min with 70% ethanol, followed by 10 min with 1% sodium hypochlorite-0.05% SDS solution, and allowed to stratify in a 0.1% agar solution in the dark for 3 d at 4 °C, prior to germination on acid-washed sand for 7 d. Seedlings were then placed in 14-L hydroponic vessels containing aerated nutrient solution composed of 1 mM $Ca(NO_3)_2$, 1 mM NaH_2PO_4, 0.75 mM K_2SO_4, 0.5 mM $MgSO_4$, 25 μM H_3BO_3, 20 μM Fe-EDTA, 2 μM $ZnSO_4$, 0.5 μM $MnSO_4$, 0.5 μM $CuSO_4$, and 0.5 μM Na_2MoO_4 (pH 6 with NaOH). Solutions were completely exchanged once per week for 4 weeks. During the final (6th) week of growth, $[K^+]_{ext}$ was dropped to 0.1 mM, and nutrient solutions were exchanged every other day. Plants were grown in a climate-controlled chamber under fluorescent lights, with an irradiation of ~200 μmol photons m^{-2} s^{-1} at plant height for 12 h d^{-1}. Temperature and humidity were controlled such as with barley (see above).

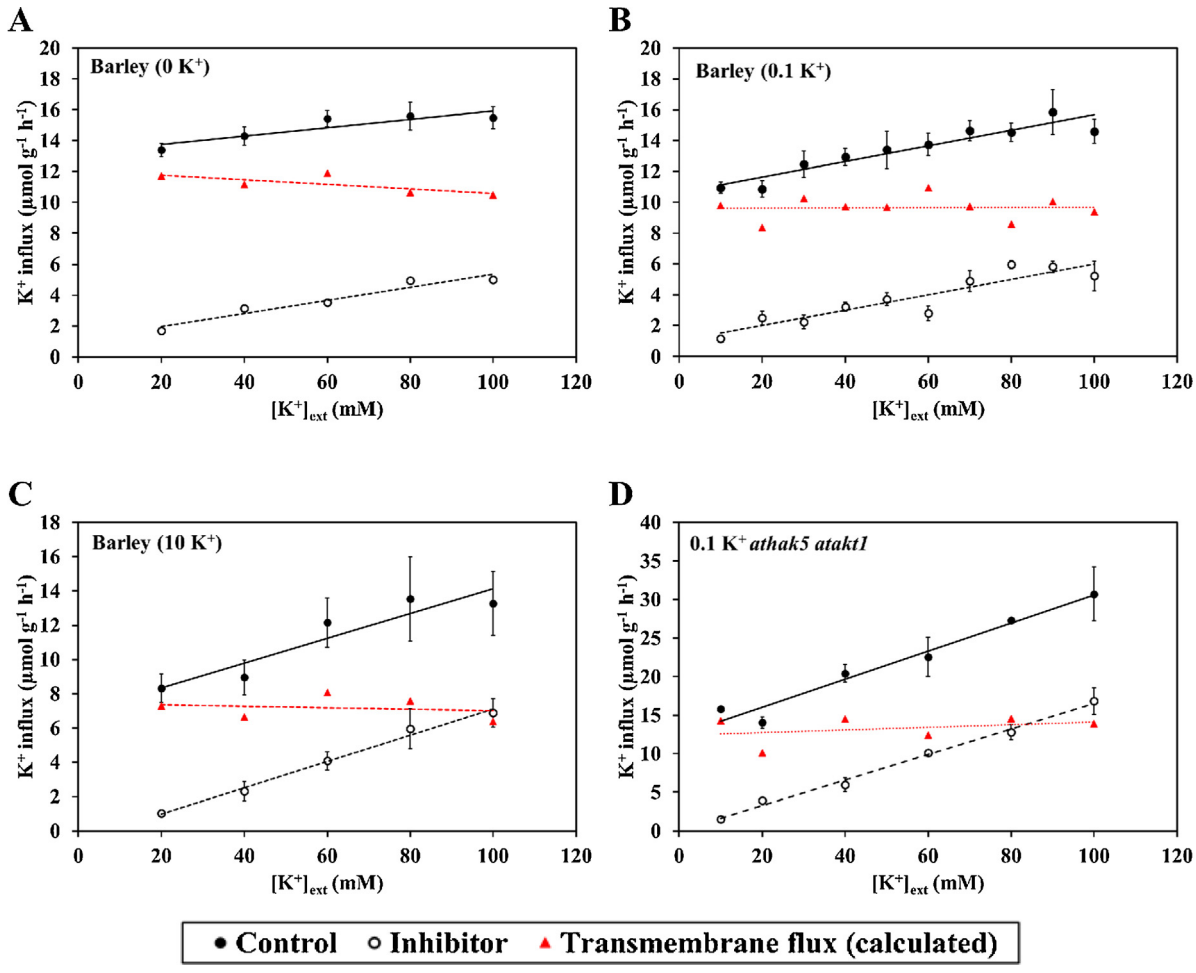

Fig. 3. Trans-membrane K^+ influx saturates in the low-affinity range, despite an apparent linear isotherm, and is inversely proportional to plant K^+ status. (A) Apparent K^+ influx in roots of intact barley seedlings between 20– 100 mM $[K^+]_{ext}$, and the effect of Cs^+ (10 mM) + TEA^+ (10 mM) at 4 °C. Seedlings were grown under nutrient-deprived conditions (200 µM $CaSO_4$ solution). Labeling time = 5 min; desorption time = 30 min. Error bars represent ± SEM ($n \geq 4$). (B and C) As in panel A, but in plants grown under full-nutrient media supplemented with 0.1 or 10 mM K^+, respectively. (D) Apparent K^+ influx with and without the presence of Cs^+ (10 mM) + TEA^+ (10 mM) + Ca^{2+} (15 mM), measured in roots of intact *athak5 atakt1* Arabidopsis seedlings. Seedlings were grown on a full-nutrient media supplemented with 1.5 mM K^+ for 4 weeks, followed by one week on 0.1 mM K^+. Labeling time = 5 min; desorption time = 5 min. Throughout, filled circles represent control fluxes, open circles represent inhibitor treatments, and red triangles represent transmembrane influx, determined as the arithmetic difference between control and treatment pairs. Error bars represent ± SEM ($n \geq 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. K^+ influx

K^+ influx in roots of intact barley and Arabidopsis was measured as described in detail elsewhere [14,38]. In brief, roots of replicate units of 3–4 plants were pre-treated for 10 min in solution either identical to growth conditions (control) or in growth solution supplemented with an increase in $[K^+]_{ext}$ and/or an inhibitory treatment. The inhibitory treatment was composed of 10 mM CsCl and 10 mM TEA-Cl (at 4 °C; in the case of barley), or 10 mM CsCl, 10 mM TEA-Cl, and 15 mM $CaCl_2$ (in the case of Arabidopsis; [13]). Roots were then immersed for a specified amount of time (1–60 min) in a solution identical to the pre-treatment solution but containing ^{42}K ($t_{½}$ = 12.36 h). From there, labeled plants were transferred to non-radioactive solution (identical to previous solutions, but excluding inhibitory treatment) for 5 s, to reduce tracer carryover, and further desorbed of radioactivity for either 5 or 30 min in fresh desorption solution (see Section 3.1). Immediately following desorption, roots were detached from shoots and spun in a low-speed centrifuge for 30 s to remove surface solution prior to weighing. Radioactivity in root and shoot tissues was counted and corrected for isotopic decay using one of two gamma counters (Packard Instrument Quantum Cobra Series II, model 5003 and PerkinElmer Wallac 1480 Wizard 3′). Throughout, all solutions were aerated, and K^+ influx is expressed in terms of µmol g^{-1} (root fresh weight) h^{-1}.

2.3. Na^+ influx

Na^+ influx (using $^{24}Na^+$ ($t_{½}$ = 14.96 h)) in roots of intact barley seedlings was measured similarly as mentioned above for K^+, only seedlings were grown on a modified Hoagland's solution (5 mM KNO_3, 0.25 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM $Ca(NO_3)_2$, 0.2 µ M Na_2MoO_4, 50 µ M $FeSO_4$, 70 µ M H_3BO_3, 14 µ M $MnCl_2$, 1 µ M $ZnSO_4$, 0.5 µ M $CuSO_4$), pH 6.3 (with KOH), and increasing concentrations of Na^+ were supplied during the experiment. Labeling time = 5 min; desorption time = 5 min.

2.4. K^+ efflux

$^{42}K^+$ efflux from roots of intact barley seedlings was examined as described previously [37,38]. In brief, roots of replicate units of 6–10 seedlings were immersed for 1 h in aerated nutrient solution containing $^{42}K^+$ (see above). From there, labeled seedlings were transferred and secured into glass efflux funnels, and roots were eluted of radioactivity with successive 13-mL aliquots of aerated, non-radioactive growth solution for 15 min (with 1-min

elution frequency). The subsequent 15 min consisted of either continued elution with growth solution (control) or growth solution supplemented with a chemical treatment (also with 1-min elution frequency). Treatments included 10 mM CsCl, 1 mM NaCN, 5 mM K_2SO_4, and pH 9.2 (with NaOH).

Immediately following the elution series, plant organs were harvested as described above for influx determination, and radioactivity from eluates, roots, and shoots was counted and corrected for isotopic decay, as described above. For comparison of $^{42}K^+$ efflux plots, the specific activities of all replicates were normalized to the arbitrary value of 2×10^5 cpm μmol^{-1}. Throughout, $^{42}K^+$ efflux is expressed in terms of cpm released g^{-1} (root fresh weight) min^{-1}.

2.5. PTS efflux

The fluorescent dye 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS; [39]) was used to trace apoplastic fluxes in roots of intact barley seedlings grown in a full nutrient solution supplemented with 0.1 mM K^+ (see above). Roots of replicate units of 6–10 plants were immersed in growth solution supplemented with 0.01% (w/v) PTS for 1 h. From there, plants were transferred and secured into efflux funnels (see above), and eluted with successive 13-mL aliquots of fresh, PTS-free, growth solution over the course of 30 min. The elution series was as follows: 1 min (five times), 2 min (once), 3 min (once), 4 min (four times). From there, plants were harvested as mentioned above, and PTS fluorescence in eluates, roots, and shoots was measured by fluorimetry ($\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm; BioTek Synergy 4).

2.6. Electrophysiology

Membrane potential differences in root epidermal and cortical cells from intact barley seedlings grown at 0.1 mM K^+ (see above) were measured as described in detail elsewhere [40]. In brief, roots were immersed in growth solution in a plexiglass cuvette mounted onto a Leica DME light microscope (Leica Microsystems Inc., Concord, ON, Canada). Root cells were impaled with a glass microelectrode, and potential differences were recorded with the use of an electrometer (World Precision Instruments Inc., Duo 773). Once sTable measurements were achieved, growth solution with various concentrations of K^+ (as K_2SO_4) was exchanged by use of peristaltic pumps at approximately 7.5 mL min^{-1} and the change in membrane potential ($\Delta\Delta\Psi$) was recorded.

2.7. Root respiration

Root respiration was measured in intact barley seedlings as described in detail elsewhere [35]. In brief, roots of 7-d-old seedlings were immersed in growth solution supplemented with various concentrations of K^+ (see text for details), in a 3-mL Hansatech cuvette/O_2 electrode system, and the decline in dissolved O_2 was recorded over 10 min, after which roots were dried and weighed, as described above (see Section 2.2).

3. Results

3.1. Low-affinity K^+ influx values are highly dependent on protocol and show no sign of saturation

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

3.2. Cellular K^+ efflux ceases in the low-affinity range and apoplastic fluxes dominate

Because declines in influx with increasing duration of labeling and desorption times are usually attributed to simultaneous tracer efflux [25,34], the efflux step in the LATS range was examined more closely. Fig. 2A illustrates the strong malleability in response to various chemical treatments, of $^{42}K^+$ efflux from roots of intact, labeled barley seedlings grown and measured under high-affinity conditions (0.1 mM $[K^+]_{ext}$). The K^+-transport inhibitor cesium (Cs^+) strongly and immediately suppressed $^{42}K^+$ efflux, as did elevated levels of externally supplied K^+ (10 mM), whereas the metabolic inhibitor cyanide (CN^-) significantly stimulated efflux. By contrast, similar treatments had no effect on efflux traces from roots of plants grown and measured under low-affinity conditions (1 and 40 mM $[K^+]_{ext}$; Fig. 2B and C, respectively). In addition, an alkaline (pH 9.2) treatment, designed to collapse the H^+ gradient across the plasma membrane, and thus disable possible H^+-K^+ exchangers [41], had no effect on efflux at 1 and 40 mM $[K^+]_{ext}$. To further test the hypothesis that $^{42}K^+$ efflux traces under LATS conditions could be apoplastic in origin, we monitored the release kinetics of the apoplastic dye 8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS; [39]) from PTS-loaded roots, and observed release patterns that were very similar to those of $^{42}K^+$ (Fig. 2D). Importantly, the half-time for PTS release from the root apoplast ($t_{½}$ = 12.8 ± 1.4 min) fell precisely into the range of half-times for K^+ release in the low-affinity range ($t_{½}$ = 10–18 min; Table 1).

3.3. Transmembrane K^+ influx saturates in the low-affinity range

We hypothesized that the linear response of influx to rising $[K^+]_{ext}$ (Fig. 1B) was the result of apoplastic tracer retention even after substantial desorption. To test this, we monitored the sensitivity of the flux to a cocktail of K^+-channel blockers, Cs^+ (10 mM) and tetraethylammonium (TEA^+; 10 mM), along with low temperature (4 °C), in order to suppress overall metabolic and transport activity [42]. In plants grown at 0.1 mM K^+ in a full-nutrient medium, influx was significantly ($P < 0.05$) inhibited by these treatments throughout the range tested (10–100 mM $[K^+]_{ext}$), but nevertheless the component of apparent K^+ influx insensitive to inhibition rose linearly with increasing $[K^+]_{ext}$ (Fig. 3B). Nevertheless, the fraction of the control flux amenable to inhibition remained surprisingly constant, near 10 μmol g^{-1} h^{-1} (Fig. 3B).

When we applied these treatments to plants that were either nutrient-deprived (grown on 200 μM $CaSO_4$; Fig. 3A) or K^+-replete (grown on a full-nutrient medium with 10 mM K^+; Fig. 3C), we observed very similar responses in influx (*i.e.* linear responses to $[K^+]_{ext}$ and a fairly uniform inhibition throughout the tested range). However, the flux amenable to inhibition was inversely proportional to K^+ supply: in K^+-starved plants, it was ~12 μmol g^{-1} h^{-1}

Table 1
Compartmental analysis in roots of intact barley (*Hordeum vulgare*) seedlings grown and measured with full nutrient media supplemented with 0.1, 1, or 40 mM K^+. Release kinetics of the apoplastic dye PTS from pre-labeled (0.1% PTS (w/v)) roots were also monitored from seedlings grown on 0.1 mM K^+.

$[K^+]_{ext}$	Influx (I)	Efflux (E)	Net flux	E:I Ratio	Half-time ($t_{½}$)
mM	$\mu mol\ g^{-1}$ (root FW) h^{-1}				Min
0.1	7.22 ± 0.23	1.86 ± 0.18	5.36 ± 0.18	0.25 ± 0.02	28.18 ± 3.40
1*	5.97 ± 0.77	0.57 ± 0.03	5.40 ± 0.77	0.11 ± 0.01	17.56 ± 1.36
40*	32.52 ± 2.88	23.91 ± 2.30	8.61 ± 0.71	0.73 ± 0.01	9.88 ± 1.20
PTS*	–	–	–	–	12.8 ± 1.4

* Measurements from the apoplast.

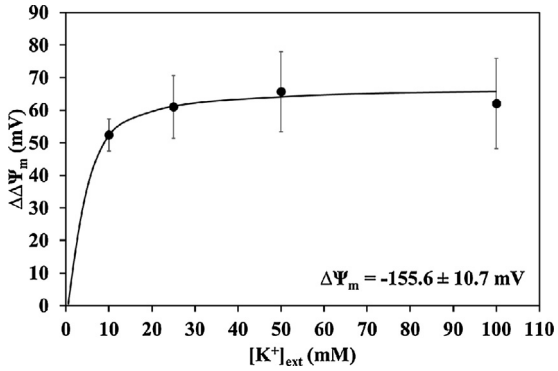

Fig. 4. Plasma-membrane depolarization saturates with rising $[K^+]_{ext}$. Depolarization ($\Delta\Delta\Psi_m$) in response to rising $[K^+]_{ext}$ in root epidermal and cortical cells of intact barley seedlings grown under a full-nutrient medium supplemented with 0.1 mM K^+. Data is fitted to a Michaelis–Menten regression analysis (K_M = 2.46 mM, V_{max} = 66.2 mV). Resting membrane potential ($\Delta\Psi_m$) = −155.6 ± 10.7 mV. Error bars represent ± SEM ($n \geq 3$).

(Fig. 3A), whereas, in K^+-replete plants, it was ~7 $\mu mol\ g^{-1}\ h^{-1}$ (Fig. 3C). In both cases, this malleable flux remained relatively constant, *i.e.* it saturated throughout the K^+ concentration range tested.

Subsequently, we tested Arabidopsis double-knock-out mutants lacking AtHAK5 and AtAKT1, the two principal K^+ transporters involved in K^+ uptake by roots [13,14]. When we applied a cocktail of the inhibitors Cs^+, TEA^+, and calcium (Ca^{2+}, at 15 mM, a concentration at which Ca^{2+} inhibits the third "back-up system", BUS; [13]), we observed nearly uniform inhibition of influx across the tested concentration range, with saturation near 13 $\mu mol\ g^{-1}\ h^{-1}$ (Fig. 3D). Importantly, in the presence of both a double genetic knock-out of K^+ transport, and a stacked transport inhibitory treatment, we nevertheless still observed a continuous linear rise of K^+ influx into plant roots (Fig. 3D).

Lastly, electrophysiological analysis was applied to K^+ transport in roots of barley seedlings, which showed a saturating depolarization in root epidermal and cortical cells over the same range of $[K^+]_{ext}$ (10–100 mM; Fig. 4). In plants grown and treated as in Fig. 3B, root plasma-membrane potentials depolarized rapidly upon exposure to 10 mM $[K^+]_{ext}$ and remained relatively constant, *i.e.* saturated, up to 100 mM. Michaelis–Menten analysis of the electrical response indicated a half-saturation constant (K_M) of 2.5 mM.

4. Discussion

This study critically re-examines the standing two-mechanism model of K^+ transport in roots, with specific focus on the nature of the linear low-affinity system. As previously reasoned and demonstrated (see [43–45], in the case of K^+; [46,47], in the case of Na^+; and [48–50], for examples from the animal literature), even a channel-mediated flux (as measured by whole-cell and patch-clamp electrophysiology) must eventually saturate with sufficiently high substrate concentration, but we nevertheless observed the classic linear response of the LATS when measured using classical radiotracer techniques, which continued even at very high substrate concentrations, up to 2 M $[K^+]_{ext}$ (Fig. S1). Clearly, fluxes at 2 M $[K^+]_{ext}$ cannot be attributable to transmembrane transport events, not only because they are energetically unfeasible (see below), but also because membrane disruption generally occurs at far lower concentrations [51]. Thus, at the maximal concentrations tested, a non-physiological, extracellular, component is almost certainly responsible for the continued linear rise in K^+ uptake. This raises a more significant question: to what extent does the apparent flux represent genuine transmembrane transport of K^+ at more moderate low-affinity concentrations (*e.g.* 10–100 mM; Figs. 1 and 3)?

To address this question, we next investigated the nature of K^+ efflux under low-affinity conditions. In our present and previous [37] analyses, we found no evidence for transmembrane efflux from barley roots to the external solution in the LATS range, under both steady-state and non-steady-state conditions, and at the low (1 mM) and higher (40 mM) end of the LATS range (Fig. 2; see also [37]). By contrast, transmembrane $^{42}K^+$ efflux was readily demonstrable in the HATS range (0.1 mM; Fig. 2A). Under non-steady-state conditions, a switch from 0.1 to 10 mM K^+ resulted in an immediate suppression of K^+ efflux to the same extent as produced by the application of Cs^+ (Fig. 2A), suggesting that efflux had been effectively silenced by the switch to a higher external K^+ concentration alone. This was further supported by the observation that efflux was equally suppressed by higher (50 mM) concentrations of K^+ (not shown). The effect of increased $[K^+]_{ext}$ on efflux provides strong *in planta* support for molecular characterizations of outward-rectifying K^+ (KOR) channels that are gated shut at elevated $[K^+]_{ext}$ [52]. Thus, as previously hypothesized [37], tracer efflux from roots under low-affinity conditions may be simply extracellular (apoplastic) in nature. A similar hypothesis has been put forward in the case of Na^+ efflux under saline conditions, based on an extensive literature review [53]. In the case of K^+, this interpretation is buttressed by the similarity in release kinetics of the apoplastic tracer PTS ($t_{½}$ = 12.8 ± 1.4 min; Table 1) and that of low-affinity K^+ release ($t_{½}$ = 10–18 min; Table 1). In agreement with our findings, Yeo et al. [39] showed that PTS release from intact rice roots can display exchange kinetics in this range ($t_{½}$ ≈22 min). It is particularly important to note, however, that with *bona fide* transmembrane K^+ fluxes, such as under high-affinity conditions (Fig. 2A), turnover rates (half-times of exchange) of the cytosolic K^+ pool can also be very similar to those of apoplastic PTS and (putatively apoplastic) K^+ pools discussed here (*e.g.* at 0.1 mM $[K^+]_{ext}$, $t_{½}$ = 28.18 ± 3.40 min; Table 1), rendering the distinction between symplastic and apoplastic fluxes highly challenging.

Because of the high efflux:influx ratios observed at high $[K^+]_{ext}$ [32] (see also Table 1), and the strong possibility that efflux under such conditions does not proceed across the plasma membrane, it is likely that unidirectional influx measurements are substantially overestimated due to the presence of a slow component of apoplastic K^+ flux. Although longer (30-min) desorption protocols can be useful for removing tracer from the apoplast (Fig. 1B), it is likely that this clearance is still not complete, given the rela-

tively long half-times of this phase of the apoplast ($t_{½}$ = 10–18 min), and thus it is important to differentiate between transmembrane and apoplastic influxes even after long desorption times. We therefore tested the effect of a range of inhibitory treatments (TEA^+, Cs^+, NH_4^+, CN^- + SHAM, and 4 °C, separately, and in combination; not shown), the most efficacious of which was a combination of Cs^+ and TEA^+ at 4 °C. Although influx was significantly suppressed using this inhibitory cocktail, we nevertheless observed a residual, non-malleable K^+ flux that maintained the strong linear response to rising $[K^+]_{ext}$ (Fig. 3), typical of LATS transport in the current model (Fig. 1A). Interestingly, similar observations of decreasing malleability of low-affinity fluxes with rising substrate supply were made in several earlier ion-transport studies. For example, Barber [54] showed that the extent of inhibition of Rb^+ and PO_4^{3-} influx, due to cold temperature (0.2 °C) or the metabolic inhibitor 2–4 dinitrophenol, decreased with rising substrate supply. Similarly, Epstein and Hagen [55] showed that Na^+ uptake under hypoxia (N_2 bubbling), although highly suppressed by the treatment, nevertheless rose significantly with increased substrate supply. Polley and Hopkins [17] showed that $^{86}Rb^+$ influx increased linearly in the presence of the strong uncoupler CCCP, at substrate concentrations up to 10 mM. Lastly, Kochian et al. [18] observed a persistent linear K^+ ($^{86}Rb^+$) influx in the presence of TEA^+, up to 10 mM Rb^+ in excised corn roots. Although it is important to note that the efficacy of inhibitors will likely not be perfect (especially in the case of competitive inhibitors where substrate (*e.g.* K^+) supply can be relatively high), such findings may be interpreted as resulting from an apoplastic flux that is unaffected by metabolic inhibitors, and which increases with rising substrate supply.

Indeed, it has been assumed that the LATS for various ions are relatively less metabolically sensitive than their HATS counterparts, as indicated by Q_{10} analyses [17,23,24,54], even though they are characterized by much higher fluxes [5] (see also Figs. 1, 3, and Fig. S1), which (if they are truly transmembrane fluxes) must ultimately depend on active, and energy-intensive, rectification of thermodynamic gradients by the plasma-membrane H^+-ATPase and other mechanisms [56]. Fig. S2 shows the estimated respiratory requirement (in terms of root O_2 consumption) for low-affinity K^+ influxes in barley seedlings, using established models for ion-O_2 flux relationships [33,56]. In this model, depolarization of the membrane electrical potential due to the thermodynamically passive, uniseriate uptake of K^+ via ion channels (*i.e.* Shaker-like AKT1 complexes and possibly NSCCs, in the case of the "back-up system" [13,57]) is compensated for by the active extrusion of protons via the H^+-ATPase (a 1 K^+:1 H^+ stoichiometry). Secondly, for every 1 H^+ pumped via the H^+-ATPase, 1 ATP molecule is consumed via hydrolysis [58], and thirdly, a stoichiometry of 5 ATP molecules synthesized for every O_2 molecule consumed in respiration is observed (*i.e.* the 'phosphorylation efficiency' or P:O_2 ratio; [33,56]). In sum, the depolarization brought about by five K^+ ions entering the cell passively, via channels, is compensated for energetically with one O_2 consumed in respiration. As shown in Fig. S2A, the respiratory O_2 demand for K^+ influx ≤100 mM $[K^+]_{ext}$ is at most ~8 μmol O_2 g^{-1} root FW h^{-1}, roughly half the total O_2 flux measured directly in roots of intact seedlings. Although still feasible at these levels, the energetic demands are pronounced, and become very problematic in the case of K^+ fluxes measured above 100 mM $[K^+]_{ext}$. By 200 mM $[K^+]_{ext}$, the O_2 flux required to compensate for a K^+ influx of >100 μmol g^{-1} h^{-1} exceeds the total energy supply of the plant (Fig. S2B), leaving no room for other respiratory demands (*e.g.* growth, maintenance, and the fluxes of other substrates). Such energetic inconsistencies were initially discovered in the case of Na^+ fluxes under salt stress, and call into question the plausibility of extremely high transmembrane ion fluxes, as have been seen with sodium [56], and here in the case of K^+. The energy demands would become even greater were one to postulate a concomitantly operating active-efflux mechanism, such as a K^+/H^+ antiporter (*e.g.* via CHX proteins; [41]), at the plasma membrane (Fig. S2; Table S2; however, see above).

Taken together, our findings strongly suggest that K^+-influx traces, as obtained by established protocols, are unlikely to accurately represent genuine transmembrane K^+ fluxes. However, a genuine membrane flux may still be estimated by subtracting the non-malleable fluxes (representing apoplastic fluxes; Fig. 3) from the uninhibited control fluxes. Interestingly, this subtraction exercise reveals a possible 'transmembrane' flux that follows Michaelis–Menten kinetics, and is saturated between 10 and 100 mM $[K^+]_{ext}$. This flux is therefore not compatible with the paradigm of an indefinitely linearly rising transmembrane influx, but exhibits a concentration-dependence pattern remarkably similar to those found in enzyme kinetics and high-affinity transport systems. Corroborating evidence for a saturable transmembrane influx is provided by electrophysiological experiments showing a saturable depolarization response in root epidermal and cortical cells in intact barley seedlings exposed to 10–100 mM $[K^+]_{ext}$ (Fig. 4). Michaelis-Menten analysis of the electrical response yields an apparent K_M of 2.5 mM, in good agreement with reported kinetic values for channel-mediated fluxes [1,7,20,21,43,59]. K_M values from tracer-uptake experiments were not obtained since transmembrane K^+ influx had already appeared to reach its maximal rate (V_{max}) by 10 mM $[K^+]_{ext}$ (Fig. 3); this apparent V_{max} ranged from 7 to 12 μmol g^{-1} h^{-1} and showed a strong dependence on the level of K^+ provision during growth (Fig. 3A–C). Thus, the intensity of transmembrane K^+ transport appears to depend on plant K^+ status in the low-affinity range, a property previously thought to be exclusive to the high-affinity range [2,3,21]. However, this variability in V_{max} probably does not reflect changes in transporter-gene expression (as is known to occur in the high-affinity range; [21]) but rather may be the result of the well-documented effects of plant K^+ status on the membrane electrical polarization that drives the thermodynamically passive, channel-mediated flux [5]. In Arabidopsis *athak5 atakt1* double-knock-out mutants, which we used in conjunction with inhibitors to bring about a more complete cessation of transmembrane K^+ transport, we still observed a linearly rising influx after inhibition. Subtraction of this inhibitor-insensitive component from the control flux revealed a transmembrane flux saturating at ~13 μmol g^{-1} h^{-1} (Fig. 3D) indicating a moderate capacity of the genetically as-yet-undefined "back-up system" (BUS) for K^+ uptake [12–14]. The relatively high transmembrane flux in the double mutants can, in part, be explained thermodynamically by the highly negative plasma-membrane potentials in their root cells (below −250 mV; [14]). Indeed, this was the explanation proposed for very high (25 μmol g^{-1} h^{-1}, at 5 mM $[K^+]_{ext}$), malleable, and short-lived K^+ fluxes observed in barley plants, upon withdrawal of NH_4^+ supply [14]. Interestingly, we observed broadly similar fluxes (with and without inhibitors) in Col-0 wildtype seedlings compared to the double mutant (not shown), suggesting that the BUS compensates (perhaps completely) for the lack of AtHAK5 and AtAKT1 in the $[K^+]_{ext}$ range tested. Although beyond the scope of this study, a thorough characterization of the BUS, involving its kinetics, selectivity, regulation, and genetic candidate(s), is warranted (see [12–14] for early indications).

Unifying influx and efflux data, we propose that, as ion concentrations rise, the increased apoplastic tracer retention and cycling can account for a persistent and increasing portion of the measured tracer fluxes, giving rise to linearly rising influx isotherms, which can indeed continue to rise *ad absurdum* (Fig. S1), to levels that are neither energetically probably nor compatible with the limits of cellular membrane integrity [51] (and references therein). Our data suggest that the extremely high LATS fluxes often reported with short labeling-time protocols [25,35,36] are largely attribuTable to apoplastic "noise", which is then multiplied many-fold when

extrapolating influx values to a per-hour flux (*e.g.* 60-fold for 1-min uptake measurements, or 30-fold for the commonly recommended 2-min protocols; [25]; Fig. 1C). It therefore became apparent that the recommendations for very short labeling and desorption times under low-affinity conditions, due to high rates of efflux from root cells (see above), are unfounded.

A new problem this proposal raises, however, concerns the structural and functional nature of this apoplastic fraction. It does not represent a component of the "Donnan space" of cell walls, for example, as it does not display ion-exchange characteristics, *i.e.* K^+ ions are not displaceable by strong divalent cations, such as Ca^{2+} or Mg^{2+} (not shown, but see Fig. 3D), and it shows desorption kinetics slower than what are usually attributed to the Donnan phase of the apoplast [60], but similar to other efflux phases verified as arising from the cytosol, at lower substrate concentrations, in other experimental works [60] (see also Fig. 2A and Table 1). Clearly, the problematic structure and function of this apoplastic phase in roots requires further investigation.

The contribution of non-transmembrane transport also pertains to long-distance transport to the shoot. Fig. S3 shows an experiment in which we stepped up the external supply of K^+ to roots of intact plants, and observed a significant rise in the contribution of $^{42}K^+$ accumulation in the shoot relative to the total accumulation in the plant. We suggest, based on inhibitory treatments (Fig. 3B), that this rising shoot flux also has a significant apoplastic component, an idea which agrees with a substantial body of literature showing there to be an "apoplastic bypass flow" from root to shoot in the low-affinity range, especially well-demonstrated in the case of Na^+ translocation in rice, but also observed with potassium and in other plant species [39,61]. Such long-distance fluxes can contribute additionally to influx isotherms in cases where whole plants are examined, and can be misconstrued as representing fractions of influx across cellular membranes. Generally, however, these are minor contributions to overall influx, as shoot translocation represents a small fraction of net accumulation in short-term tracer studies, and is typically small compared to total unidirectional influx and efflux in roots (as manifest in the high efflux:influx ratios at high substrate concentrations; see above). The largest contributor to misattributed influxes in the LATS range, therefore, is an apoplastic "bypass" within the root system itself, which has, hitherto, gone unnoticed. In further support of this idea, some of the highest LATS-range ion fluxes on record (Na^+ fluxes in Arabidopsis), were also obtained in excised roots, lacking a shoot system altogether [25]. We have previously noted that it is unlikely that these fluxes proceed across the plasma membrane, on energetic grounds [56].

In summary, we propose to revise the model for K^+ transport in roots of higher plants (Fig. 5). Due to the negligible contribution of apoplastic fluxes at low $[K^+]_{ext}$ (*e.g.* <1 mM), it is clear that tracer-influx measurements accurately reflect the activities of high-affinity transporters (*e.g.* AtHAK5 and HvHAK1) in this range. Under intermediate LATS conditions (*i.e.* 1–10 mM $[K^+]_{ext}$), the activities of low-affinity channels (*e.g.* AtAKT1, HvAKT1, and BUS) mediating transmembrane influx can also be gauged by tracer analysis, but only if distortions brought about by apoplastic tracer retention are taken into account. Some contribution to transmembrane influx in this range might also occur via high-affinity transporters (*e.g.* AtHAK5 or HvHAK1), which can display dual-affinity kinetics [62]; however, their contribution is likely to be negligible, given that the prevailing thermodynamic conditions favor passive influx across the membrane (Table S2), and that the expression of these transporters is down-regulated even at moderate $[K^+]_{ext}$ [4]. Under high LATS conditions (*i.e.* 10–100 mM $[K^+]_{ext}$), however, the standing model needs major revision, with transmembrane systems becoming saturated, and linear apoplastic fluxes beginning to dominate (Fig. 5). It is critical to recognize the

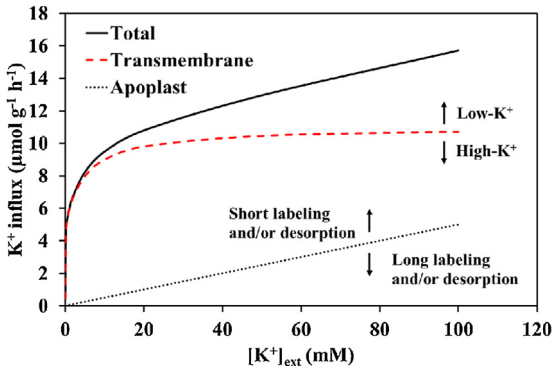

Fig. 5. The revised model of K^+ acquisition in roots of higher plants. The apparent flux measured with radiotracer (solid black line) is composed of a saturable transmembrane flux (red dashed line) and a linear extracellular (apoplastic) flux (black dotted line). The magnitude of the transmembrane flux is regulated by plant K^+ status. The apoplastic contribution, by contrast, is strongly dependent on measurement protocol (*i.e.* labeling and desorption time). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contribution of apoplastic fluxes in this range, in order to accurately discern transmembrane events and attribute them to the expression of specific genes and the activities of specific gene products.

Lastly, but perhaps most significantly, we believe that our model may also pertain to the linear nature of low-affinity fluxes for other ions, such as NH_4^+, NO_3^-, and Cl^-, and, most importantly, Na^+, in the context of salinity stress. Na^+ influx into plant roots has been shown to display a strong linear response to external $[Na^+]$ throughout the saline range in several studies [29] (and references therein; see also Fig. S4), but, if the arguments presented here for K^+ (and elsewhere; [56]) apply to Na^+ as well, such fluxes may also be found to be apoplastic [53]. Indeed, we believe that the lack of progress in identifying transporters for Na^+ in the saline range [29] is, in part, related to a persistent misattribution of Na^+ fluxes to transmembrane events. The demonstration of a substantial apoplastic Na^+-transport artefact could lead the way to the discovery of genuine membrane transport mechanisms in this critically important case.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.12.003.

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