ng (complex pools) to 0.5 ng (pure clone) per oocyte]. Oocytes were incubated for 24 to 48 hours, treated with coelenterazine [10 μM coelenterazine (Molecular Probes, Eugene, OR) and 30 μM reduced glutathione in OR-2 media (no calcium)] for 2 to 3 hours with gentle orbital shaking in the dark at 18°C, and returned to ND-86 medium with calcium (maintained in the dark with shaking) until measurements were initiated. For luminometer measurements, oocytes (singly or in pairs) were transferred to plastic tubes (75 mm by 12 mm, Sarstedt) containing 2.9 ml of Ca^{2+}-free OR-2 medium. Each cRNA pool was tested in triplicate. Measurements (duration 2 min) were triggered by the injection of 0.1 ml of 30 μM MK-0677.

19. Additional GHS-R clones from the swine cDNA library were identified by hybridization of the clone 7-3 ^{32}P-labeled insert to slot-blot pools of plasmid DNA (500 cDNAs per pool). Filters were prehybridized [at 42°C for 4 hours in 5× standard saline citrate (SSC) with 5× Denhardt's solution, 250 μg/ml of tRNA, 1% glycine, 0.075% SDS, 50 mM $NaPO_4$ (pH 6), and 50% formamide], and hybridizations were done at 42°C for 20 hours in 5× SSC, with 1× Denhardt's solution, 0.1% SDS, 50 mM $NaPO_4$, and 50% formamide. Clonal isolates were identified by colony hybridization. Human pituitary homologs of the swine GHS-R were obtained by screening a cDNA library [lambda ZAP II (Stratagene); ~2 × 10^6 phages gave 21 GHS-R clones]. DNA sequencing was done on both strands [automated Applied Biosystems instrument (ABI model 373); manually by dideoxy chain termination (Sequenase version 2.0; U.S. Biochemical, Cleveland, OH)]. Database searches [GenBank 92, EMBL 43, Swiss-Prot 31, PIR 45, dEST (Gbest 92), and Prosite 12], sequence alignments, and analysis of the GHS-R nucleotide and protein sequences were done with the GCG Sequence Analysis Software (Madison, WI; pileup, peptide structure and motif programs), FASTA and BLAST search programs, the PC/Gene software suite from Intelligenetics (San Francisco, CA; protein analysis programs), and Lasergene software (DNA Star, Madison, WI).
20. We transfected COS-7 cells with GHS-R expression plasmids by using lipofectamine (Gibco-BRL) as described. Binding of [^{35}S]MK-0677 (~1000 Ci/mmol) was done with control swine pituitary membranes, membranes from mock transfected cells, and crude membranes prepared from transfected cells (*12*). Crude cell membranes were prepared on ice at 48 hours after transfection. Binding reactions were done at 20°C for 1 hour in a total volume of 0.5 ml containing 0.1 ml of membrane suspension (25 μg of protein), 10 μl of [^{35}S]MK-0677 (0.05 to 1 nM), 10 μl of competing drug, and 380 to 390 μl of homogenization buffer. Specific binding (>90% of total) equaled the difference between total and nonspecific binding obtained in the presence of 50 nM unlabeled MK-0677.
21. Rhesus monkeys were euthanized, and the brains were removed and immediately frozen in isopentane on dry ice at −35°C and stored at −70°C. Coronal or sagittal sections (~10 μm) were cut in a cryostat (Reichert) at −18°C to −20°C. Sections were thaw-mounted on "Probe On" slides (Fisher Scientific), air-dried for approximately 1 hour, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 5 min, rinsed in PBS for 2 min, defatted and dehydrated in an ethanol series (50, 70, and 95% for 5 min each), and stored in 95% ethanol at +4°C. The 3′ end–labeled probes, specific for the type Ia human GHS-R (specific activity ~1.5 × 10^9 cpm/μg), were each 45 bases long and antisense to nucleotides 855 through 909 and 979 through 1023. Hybridizations of rhesus brain sections were done as described [D. J. S. Sirinathsinghji *et al.*, *Neuroscience* **34**, 675 (1990); D. J. S. Sirinathsinghji and S. B. Dunnet, in *Molecular Imaging in Neuroscience*, N. Sharif, Ed. (Oxford Univ. Press, Oxford, 1993), p. 43]. After hybridization, the sections were washed for 1 hour in 1× SSC at 57°C, briefly rinsed in 0.1× SSC and dehydrated in 70% and 95% ethanol, air-dried, and then exposed to Hyperfilm β-max x-ray film (Amersham) for 7 days. Adjacent slide-mounted sections incubated with labeled oligonucleotide probe in the presence of a 100-fold excess of unlabeled oligonucleotide probe or with a sense probe from the same region produced no hybridization signal.
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25. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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TECHNICAL COMMENTS

High-Affinity Potassium Uptake in Plants

Plant roots accumulate K^+ against its electrochemical gradient from the micromolar amounts that prevail in most soils (*1*). Francisco Rubio *et al.* (*2*) elegantly demonstrate that the high-affinity K^+ uptake transporter HKT1 from wheat roots functions as a Na^+-coupled cotransporter when expressed in yeast or *Xenopus* oocytes. Energization could therefore be provided by an inwardly directed electrochemical gradient for Na^+ across the plasma membrane, as is the case in some aquatic species (*3*).

We evaluated the possibility that Na^+-coupled K^+ transport comprises the major route for K^+ absorption in intact K^+-starved wheat roots using an electrophysiological and a radiometric approach. Addition of micromolar K^+ (or Rb^+ or Cs^+) induced marked membrane depolarizations (Fig. 1), which are typical of high-affinity transport (*4*) and absent in non-K^+–starved plants. However, depolarizations occurred in the effective absence of Na^+ and were indifferent to the presence of 1 mM Na^+. Then we tested high-affinity unidirectional uptake of the K^+ analog Rb^+ for its Na^+ dependence. Na^+ did not stimulate uptake and may even have inhibited it (Table 1).

To examine whether induction of Na^+-coupled K^+ transport requires previous exposure to Na^+ or whether it could be derepressed by relative nonavailability of H^+ for K^+:H^+ symport, we grew plants in the presence of 1 mM Na^+ or at pH 9. In each case only Na^+-independent, high-affinity K^+ and Rb^+ uptake was observed.

In situ hybridization showed that *HKT1*

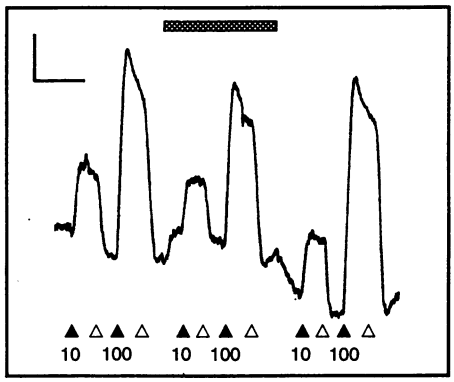

Fig. 1. Representative example of a trace showing the effect of external K^+ on the electric potential at the root surface of wheat. Wheat cultivar Atlas 66, the original source of *HKT1*, was starved of K^+ for 6 days and grown on 1 mM $CaCl_2$ at pH 6, in the presence of 1.0 mM Na^+. Closed symbols denote addition of 10 or 100 μM K^+, respectively. Open symbols denote washout of K^+. Hatched bar at the top indicates the presence of 1 mM Na^+. Horizontal and vertical scale bars are 50 s and 10 mV, respectively. Solutions were freshly prepared, and all experimental treatment was carried out in plastic to avoid Na^+ contamination. Measurements shown were made 0 to 5 mm from the root tip.

Table 1. Unidirectional Rb^+ influx (μmol·gFW^{-1}·hour^{-1}) into roots of wheat (cv. Maris Dove). Plants were grown at pH 6 with or without 1 mM Na^+, and the external Rb^+ was 50 μM. Values are the mean ± SEM of three independent determinations. Solutions were freshly prepared, and all experimental treatment was carried out in plastic to avoid Na^+ contamination.

Solution	Na^+ concentration in uptake buffer (μM)				
	0	20	50	150	500
(Minus Na^+)	2.04 ± 0.06	2.00 ± 0.05	2.14 ± 0.06	2.00 ± 0.14	1.61 ± 0.20
(Plus Na^+)	1.64 ± 0.07	1.48 ± 0.03	1.50 ± 0.10	1.62 ± 0.20	1.68 ± 0.12

is expressed mainly in the inner, cortical regions of the root (*5*), where it could function as a scavenging system to recover K^+ that leaks out of root cells into the apoplast within the root tissue (for example, through outwardly rectifying K^+ channels). However, insertion of microelectrodes into cortical cells showed depolarizations by micromolar K^+ of similar amplitude to those discussed above and these depolarizations are similarly independent of external Na^+.

Our observations demonstrate that Na^+ is not essential for high-affinity K^+ absorption in wheat roots, and hence it seems unlikely that HKT1 can make up the major transport system responsible for such uptake. It is therefore more plausible that—as was observed in *Arabidopsis thaliana* (*6*)—the principal pathway for high-affinity K^+ absorption from the soil by wheat is mediated by H^+-coupled transport.

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Response: In our report, we showed that the high-affinity wheat K^+ uptake transporter HKT1 functions as a Na^+-K^+ cotransport system (*1*). Furthermore, at toxic Na^+ concentrations, K^+ uptake by HKT1 is inhibited and low-affinity Na^+ uptake occurs, which correlates to Na^+ toxicity in plants. With the use of yeast as a screening system, we further developed a genetic strategy to isolate *HKT1* point mutations that confer Na^+ tolerance in yeast (*1*). The increased Na^+ tolerance of the isolated *HKT1* mutants was shown to be the consequence of a reduction in Na^+ uptake and decreased inhibition of K^+ uptake by toxic Na^+ concentrations (*1*). For reducing overall Na^+ influx into plant cells, this biophysical and genetic approach could be applied to other Na^+ uptake pathways in plants because many parallel pathways for Na^+ uptake exist. The cloning of *HKT1* (*2*) enables analyses of HKT1 homologs and new gene families involved in plant K^+ nutrition.

Walker *et al.* do not question these results, but with foresight go on to address effects of Na^+-coupled K^+ transport in roots. They state that (i) Na^+-cotransport cannot entirely account for high-affinity K^+ absorption in roots, (ii) that Rb^+ flux and membrane potential recordings allow quantitative analysis of the underlying transport mechanism, (iii) that one major pathway exists for high-affinity K^+-absorption from the soils, and (iv) that this is H^+-K^+ transport. We agree with (i) but not with (ii) or (iii), and (iv) has not been substantiated by other laboratories.

Plants encounter many different environmental conditions and likely have multiple mechanisms for high-affinity K^+ uptake. Na^+-coupling is not the only mechanism for Rb^+ absorption. The ability to quantitatively dissect transport properties of HKT1 (*1*, *2*) has led to recent results that show that the classical experimental methods used by Walker *et al.* are biophysically insufficient to draw the other conclusions about K^+ absorption listed, as described below.

We observed large Na^+-coupled K^+ inward currents in *Xenopus* oocytes (*1*), but Na^+-coupled Rb^+ inward currents were smaller than the resolution limit, showing an average K^+ to Rb^+ selectivity of >35 to 1 (Fig. 1A) (*4*). Even in yeast, which can compensate for the low Rb^+ conductance because of large proton pump-mediated hyperpolarizations and because of the high membrane resistance of single cells, the K^+ uptake rate through HKT1 is 15-fold larger than the Rb^+ uptake rate (*4*). Therefore, in Rb^+ flux studies in native tissue (*3*), HKT1 activity cannot be detected because the transport of Rb^+ by HKT1 will be masked by Rb^+ transport through other transporters, known to exist in plants with a lower K^+ to Rb^+ selectivity (*5*) than HKT1.

Walker *et al.* describe another classical technique of measuring depolarizations in response to micromolar K^+ that has been instrumental for measuring K^+ interactions with high-affinity systems (*6*). However, membrane depolarizations should not be interpreted as showing cation uptake, as assumed by Walker *et al.* Similar to their results, we found nonspecific wheat root membrane depolarizations in response to micromolar amounts of K^+, Cs^+, Rb^+ (Fig. 1B, $n = 31$), and Na^+. Why are high-affinity depolarizations not K^+-specific? Similar to intact roots (Fig. 1B), in HKT1-expressing oocytes, K^+, Cs^+, Rb^+, and Na^+ induce nonspecific membrane depolarizations (Fig. 1C). But with the exception of Na^+, these depolarizations were attributed to inhibition of outward current by HKT1, rather than to stimulation of cation inward currents (*4*). Even the large depolarizations in Fig. 1C do not allow one to distinguish that K^+ alone only inhibited outward currents by HKT1 (*1*, *2*), while large inward currents were stimulated by Na^+ (Fig. 1C) (*1*, *4*). The low specificity among cations in inhibiting HKT1-mediated outward currents has been previously demonstrated [figure 1D in (*2*)]. Root cortical cell membranes are an electrically complex system, containing pumps, channels, transporters, and multiple cell-cell conductances. Membrane depolarizations clearly do not allow dissection of the functional mechanisms underlying high-affinity K^+ uptake.

HKT1 function has been studied in heterologous systems where the expression is constitutive. When one is studying *HKT1* function in native tissue, one needs to verify the expression of HKT1. With the use of competitive PCR, we have found that *HKT1* mRNA is greatly (≈10- to 50-fold) induced in 6-day-old seedlings by complete nutrient starvation for 2 days, and strongly suppressed in the presence of 1 mM K^+ together with other cationic nutrients (*7*). Walker *et al.* assume that HKT1 is expressed under the conditions employed for their experiments.

To draw their conclusions, Walker *et al.* implicitly assume that there is one major pathway for high-affinity K^+ uptake in plants. But our finding that HKT1 is a Na^+-coupled transporter (*1*) suggests to us that other high-affinity K^+ transporters with other transport mechanisms are likely to exist. Recent molecular biological studies have hinted at the large extent of nutrient transporter gene families (*8*, *9*). The limited genetic identification of plant Na^+-excluding Na^+-tolerant plants points to the fact that redundant pathways for Na^+ uptake also exist in plant roots (*10*, *11*). HKT1 may play an important role in K^+ nutrition under conditions of low K^+ and sufficient Na^+ in soil. Under these conditions, Na^+ stimulates root growth and K^+ nutrition [(*12*), and references therein]. A recent study of *Arabidopsis* has shown doubling of plant growth by weight in the presence of Na^+ (*13*).

Walker *et al.* further suggest that the major mechanism for high-affinity K^+ uptake in plants is H^+-coupled K^+ uptake. They did not analyze whether Rb^+ uptake was pH-dependent or not. Because some H^+-coupled transporters can function as Na^+-coupled transporters and vice versa, we agree with the hypothesis that H^+-coupled K^+ transporters provide one of the mechanisms for K^+ uptake in plants. We found that the activity of HKT1 was not

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