Title
SLAC1-related signal transduction pathway involved in ABA-induced stomatal closure and K⁺ selective transport by the OsHKT2;4 transporter from rice (Oryza sativa) with atypical Na⁺ transport properties and competition in permeation of K⁺ over Mg²⁺ a...

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SLAC1-related signal transduction pathway involved in ABA-induced stomatal closure and

K⁺ selective transport by the OsHKT2;4 transporter from rice (Oryza sativa) with atypical Na⁺ transport properties and competition in permeation of K⁺ over Mg²⁺ and Ca²⁺ ions

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Dennis E. Brodsky

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2011
The thesis of Dennis E. Brodsky is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
DEDICATION

Dedicated to my family for their constant support, as well as to those that challenged me along the way.
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SLAC1-related signal transduction pathway involved in ABA-induced stomatal closure and K⁺ selective transport by the OsHKT2;4 transporter from rice (Oryza sativa) with atypical Na⁺ transport properties and competition in permeation of K⁺ over Mg²⁺ and Ca²⁺ ions

by

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University of California, San Diego, 2011

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ABA is a phytohormone known to play a major role in plant development and the plant stress response. SLAC1, the first anion channel discovered in plants, lies towards the end of the ABA signaling pathway. To analyze SLAC1-related ABA signaling, anion channel activity was measured in Xenopus laevis oocytes. Certain protein kinases were able to activate SLAC1 activity in oocytes, and the PP2C phosphatase ABI1 was able to inhibit these kinase-
activated SLAC1 currents. When ABA and receptor PYR1 were added to the above components, SLAC1 activity was restored due to putative inhibition of ABI1. Mutations in SLAC1 and ABI1 had notable effects on SLAC1 channel activity in oocytes.

Na\(^+\) and K\(^+\) homeostasis plays a crucial role in plant development and growth. A major group of plant Na\(^+\) and Na\(^+\)/K\(^+\) transporters involved in salinity tolerance is the HKT family. HKT transporters are characterized into two major classes, based on structure as well as ion selectivity. The ion selectivity of class II OsHKT2;4 from *Oryza sativa* rice was studied in yeast and oocytes, and its localization was identified in Arabidopsis mesophyll protoplasts. Unlike typical class II HKT transporters, OsHKT2;4 is believed to be a K\(^+\) transporter with little dependence on Na\(^+\). OsHKT2;4, and TaHKT2;1 from wheat were found to be permeable to divalent cations. Cation competition experiments demonstrated that OsHKT2;4 and TaHKT2;1 have a higher selectivity for K\(^+\) or Na\(^+\)/K\(^+\) respectively over these divalent cations, suggesting that Ca\(^{2+}\) and Mg\(^{2+}\) transport is small and dependent on plant K\(^+\) concentrations *in vivo*. 
I.

SLAC1-related signal transduction pathway involved in ABA-induced stomatal closure
1.1 Abstract:

The phytohormone abscisic acid (ABA) is produced in response to abiotic environmental stresses and plays a key role in inducing stomatal closure. One of the key components of the ABA-induced stomatal closing pathway is SLAC1 (Slow Anion Channel-Associated 1), a guard cell specific S-type anion channel that is responsible for the efflux of anions, which is required for ABA-, CO$_2$-, and Ca$^{2+}$- induced stomatal closing. In *Xenopus laevis* oocytes, SLAC1 activity has been observed in the presence of activating kinases such as CPK23 (Calcium-Dependent Protein Kinase 23) and OST1 (Open Stomata 1). Several members of the PP2C (type 2C protein phosphatase) family, including ABI1 (ABA Insensitive 1), have been shown to inhibit SLAC1 activity in oocytes, due to putative interaction with kinases necessary for SLAC1 activation. Recently, ABA has been shown to bind to the ABA receptor PYR1 (Pyrabactin Resistance 1) and cause structural changes that allow the interaction of PYR1 with ABI1, and thereby inhibiting the activity of ABI1. In my research, this ABA signaling pathway was reconstituted and analyzed in the *Xenopus* oocyte expression system. Mutations in either SLAC1 or ABI1 were found to have prominent effects on SLAC1 channel activity in oocytes.
1.2 Introduction:

Stomata, present in the epidermis of most plants, provide gates for regulating carbon dioxide and water exchange between the plant and the atmosphere. The stomatal pores, formed by two surrounding guard cells, open and close in response to environmental factors to maintain homeostasis within the plant. Stomata in plants must balance the uptake of carbon dioxide for photosynthetic carbon fixation with the loss of water due to transpiration. Control of stomatal aperture is maintained through a variety of signals, including humidity, light, CO$_2$, ozone (Schroeder et al. 2001), and phytohormones (Hubbard et al. 2010; Kim et al. 2010). Stomatal aperture is controlled by the transport of osmotically active ions and organic metabolites, including potassium, chloride, and malate, across the guard cell membrane (Schroeder et al. 1987; Schroeder and Hagiwara 1989; Vahisalu et al. 2008).

Water is crucial for plants, as it provides a source of electrons during photosynthesis, serves as a solvent to allow minerals into the plant, and allows the transport of metabolic products throughout the plant. A prominent phytohormone that mediates stomatal closure is abscisic acid (ABA), which is produced during conditions of drought in order to limit the negative effects of excessive transpiration. During times of elevated ABA production, guard cell turgor pressure is reduced by the efflux of potassium ions and anions, as well
as the gluconeogenic conversion of malate into starch. (MacRobbie 1998; Kim et al. 2010). ABA causes an increase in guard cell intracellular [Ca] ([Ca]) (McAinsh, Brownlee, and Hetherington 1990; Assmann 1993; MacRobbie 2000; Pei et al. 2000), which leads to the down-regulation of inward-rectifying K\(^+\) channels, and activation of both slow-sustained (S-type) and rapid-transient (R-type) anion channels (Schroeder and Hagiwara 1989; Schroeder and Hagiwara 1990; Linder and Raschke 1992; Schroeder and Keller 1992; Pei et al. 1997; Siegel et al. 2009). Depolarization of the membrane, caused by the efflux of anions, precedes the activation of outward-rectifying K\(^+\) channels, further lowering turgor pressure in the guard cells and leading to stomatal closure (Schroeder JI, Raschke K, and Neher E 1987; Schroeder 1988; Thiel G, MacRobbie EAC, Blatt MR 1992; Hosy et al. 2003). In addition, ABA is believed to induce stomatal closure through both calcium dependent and independent pathways (Gilroy et al. 1991; Allan et al. 1994; Levchenko et al. 2005).

Anion channels have been proposed to function as crucial components of the stomatal response to physiological and stress stimuli. Perhaps the first of its kind was an isolated *Arabidopsis* gene SLAC1 (Slow Anion Channel-Associated 1), a slow anion efflux channel that is localized to the guard cells in leaves, and regulates stomatal aperture in response to various environmental stresses.
Originally identified among several ethyl methanesulphonate (EMS) mutants in a screen for Arabidopsis O₃ sensitivity, *rcd3* (radical-induced cell death 3) was renamed to *slac1* upon discovery of its profound effects on stomatal aperture (Vahisalu et al. 2008). SLAC1 mutant plants have been shown to display hindered stomatal closure in response to elevated humidity, ABA, CO₂ levels, ozone, nitric oxide, and calcium, suggesting that SLAC1 is downstream of these signals and crucial to stomatal closure (Vahisalu et al. 2008). Patch clamp recordings in guard cell protoplasts showed that while S-type anion channel activity was inhibited in *slac1* mutants, R-type anion channels were not affected (Vahisalu et al. 2008). Transgenic plant studies with SLAC1 fused to green fluorescent protein (GFP) demonstrated that SLAC1 is localized to the plasma membrane of guard cells (Negi et al. 2008). SLAC1 was shown to participate in K⁺, Cl⁻, malate, and fumarate efflux; not surprising results considering the similarity of SLAC1 to the C4-dicarboxylate transporter family (Saier et al. 1999; Negi et al. 2008). After an atomic-resolution crystal structure was solved for a bacterial anion channel that is a SLAC1 homologue in *Haemophilus influenzae*, TehA, structural analysis of SLAC1 led to the conclusion that the channel is a trimeric protein with ten transmembrane helices arranged in helical-hairpin pairs to form a five-helix transmembrane pore that is gated by a crucial phenylalanine residue (Chen et al. 2010).
Elements of the ABA signaling pathway have been discovered through genetic screens, with certain mutants revealing an ABA-insensitive phenotype. Among these elements are protein phosphatases from the PP2C (type 2C protein phosphatase) family and the SnRK2 kinases (Snf1-related protein kinase); especially SnRK 2.6, also known as OST1 (Open Stomata 1) (Leung 1994; Meyer, Leube, and Grill 1994; Mustilli 2002). Members of the PP2C family have been shown to deactivate SnRK kinases through dephosphorylation activities in vitro (Umezawa et al. 2009; Vlad et al. 2009). Using protein-protein interaction assays, the PP2C ABI1 as well as OST1 were found to be significant regulators of SLAC1 in the ABA signaling pathway. In vitro kinase assays demonstrated that CPK23 (Calcium-Dependent Protein Kinase 23) and OST1 directly phosphorylate SLAC1, and certain point mutations in the phosphorylation sites can reduce the degree of phosphorylation of the anion channel (Geiger et al. 2009; Geiger et al. 2010; Vahisalu et al. 2010). Patch clamp analyses of CPK23, OST1, and SLAC1 mutant Arabidopsis guard cell protoplasts indicated that these genes were crucial for anion efflux (Geiger et al. 2009, Geiger et al. 2010).

Recently, an apex of the ABA signaling pathway has been discovered in the form of an ABA receptor named PYR1 (Pyrabactin Resistance 1), part of the START protein family, which share a conserved hydrophobic ligand-binding pocket (Radauer, Lackner, and Breiteneder 2008; Park et al. 2009;
Lee et al. 2010). In addition to PYR1, there have been 13 genes in the Arabidopsis genome with similarity to PYR1, named PYL1-PYL13 (PYR1-Like). This entire 14 member gene family has been named RCAR1-RCAR14 (Regulatory Component of ABA Receptor). The PYR/PYLs have been shown to bind to phosphatases from the PP2C family, and inhibit the activity of these phosphatases in the presence of ABA (Park et al. 2009; Nishimura et al. 2009; Nishimura et al. 2010).

Xenopus oocyte electrophysiology experiments demonstrated that SLAC1 in the presence of protein kinases OST1, CPK23, and, to a lesser extent, CPK21 and CPK6 are able to demonstrate anion channel activity (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010). SLAC1 that has been activated in oocytes by OST1 or CPK23 has been shown to be repressed by the PP2C-type phosphatases ABI1, ABI2 (Geiger et al. 2009; Geiger et al. 2010), and PP2CA (Lee et al. 2009).

In my study, I have observed SLAC1 activation in response to several of the protein kinases including OST1 and CPK23, with and without the presence of the split YFP system, in Xenopus oocytes. I have also observed the strong inhibition of SLAC1 activity in the presence of phosphatases of the PP2C family. I have reactivated the SLAC1 anion channel in Xenopus oocytes by expressing the PYR1 receptor, in order to inhibit PP2C activity in the
presence of ABA, and therefore restore SLAC1 anion channel expression. I have observed hindered SLAC1 activity when potential phosphorylation sites, amino acids serine and threonine, of the anion channel were mutated to glycine, which abolishes the possibility of phosphorylation. When the serines and threonines of interest were mutated into aspartate to mimic phosphorylation events, the resulting inward currents were more similar to activated wild type SLAC1. Finally, two tested point mutations in ABI1 were able to affect the activity of the PP2C phosphatase, by either preventing ABI1 activity with kinases, or hindering the interaction of ABI1 with PYR1.

1.3 Materials and Methods:

Construct preparation and electrophysiology in *X. laevis* oocytes

All constructs were cloned into pNB1 oocyte expression vector (Nour-Eldin et al. 2006). cRNA was prepared using the mMessage mMACHINE transcription kit from Ambion.

Approximately 50-100 ng of cRNA, in a total volume of 50 nl, was injected into each oocyte for voltage clamp recordings. The recordings were performed 2-3 days after injection, with a Cornerstone (Dagan; Minneapolis, MN) TEV-200 two-electrode voltage clamp amplifier. Data analyses were
performed using an Axon Instruments Digidata 1440A Low-Noise Data Acquisition System (Molecular Devices; Sunnyvale, CA).

Oocytes were subjected to voltage pulses, with a holding potential of 0 mV, using a voltage protocol with a range of -180 mV to +40 mV in 20 mV increments, followed by a -120 mV voltage pulse, as used previously (Geiger et al. 2009). Data were low-pass filtered at 20 Hz throughout all the recordings. Oocytes were bathed in 75 mM NaCl, 20 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, with pH = 7.4 (using Tris base) and osmolarity balanced to 220-260 mosmol/kg with D-mannitol for the recordings, unless otherwise mentioned. The oocytes were penetrated with electrodes filled with 3 M KCl. For the oocyte groups with injected ABA, 500 µM ABA was injected into each oocyte (to a final concentration of approximately 50 µM ABA, assuming 500 nl total volume in the oocyte), performed 15-30 minutes prior to the voltage clamp experiments. Error bars indicate standard error of the mean. All experiments were performed at room temperature.

1.4 Results:

SLAC1 in an ABA-Related Signaling Pathway in X. laevis oocytes

Two-electrode voltage clamp experiments in X. laevis oocytes were performed to analyze the anion transport properties of SLAC1. When SLAC1 cRNA was injected into oocytes alone, the resulting inward currents were only
Figure 1.1. (A) SLAC1 is activated by protein kinases OST1 and CPK23 in *Xenopus* oocytes. Kinases SnRK2.3 and CPK31, homologues of OST1 and CPK23 respectively, are not as effective at activating the anion channel. (B) During hyperpolarized voltage conditions (-180 mV is shown), SLAC1 is active in the presence of OST1 and, to a larger extent, CPK23.
slightly larger than those of water-injected control oocytes (Fig. 1.1). However, in the presence of protein kinases OST1 and especially CPK23, SLAC1 activity was greatly increased (Fig. 1.1), as observed in recent literature (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010). Other kinases SnrRK2.3 and CPK31, homologues of OST1 and CPK23 respectively (Cheng et al. 2002; Hrabak et al. 2003), were not able to enhance SLAC1 activity (Fig. 1.1). When OST1 and CPK23 were injected in the absence of SLAC1, no significant inward currents were detected, similar to the water control oocytes (Fig. 1.1). This was also observed in literature (Geiger et al. 2009; Geiger et al. 2010).

When the PP2C phosphatase ABI1 was injected along with SLAC1 and OST1 or CPK23, the inward current was strongly inhibited (Fig. 1.2). This result agrees with recent literature, in which SLAC1 currents were abolished in the presence of ABI1, as well as other phosphatases of the same family (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010).

The next step was to see the upstream effect of the ABA receptor PYR1 on the SLAC1 current inhibited by ABI1. In the absence of ABA, PYR1 demonstrated a negligible effect on the SLAC1 currents (Fig. 1.3). However, in the presence of ABA, the addition of PYR1 resulted in a slight increase in SLAC1 currents (Fig. 1.3).
Figure 1.2. (A) In the presence of a phosphatase from the PP2C family, ABI1, the activation of SLAC1 by either OST1 or CPK23 was inhibited. (B) During hyperpolarized voltage conditions (-180 mV is shown), the extent of the deactivation can easily be seen.
Figure 1.3. (A, C) The ABA receptor PYR1 alone has no effect on the oocytes in which SLAC1 activity was inhibited by ABI1. After injecting the oocytes containing PYR1 with ABA 10-30 minutes prior to recording, slight reactivation of SLAC1 was observed at voltage levels more negative than -140 mV. (B, D) At -180 mV, the extent of PYR1/ABA activity on the deactivated SLAC1 currents can be seen.
To enhance the interaction between SLAC1 and OST1 or CPK23, split YFP was attached to the two components of interest. SLAC1 was attached to the C-terminal end of the split YFP, and OST1 or CPK23 was attached to the N-terminal end of the split YFP. In both cases, the split YFP fragment was attached to the C-terminal end of the gene of interest. In the presence of the split YFP system, the interaction of SLAC1 and OST1 resulted in much larger inward currents (Fig. 1.4). On the other hand, the interaction between SLAC1 and CPK23 with split YFP resulted in approximately the same size currents as without split YFP (Fig. 1.4). ABI1 was still effective at inhibiting SLAC1 currents in the presence of split YFP, with SLAC1 activation from either CPK23 or OST1 (Fig. 1.4).

Since the currents were much larger with SLAC1 and OST1 with split YFP, the effect of PYR1 on ABI1-inhibited SLAC1 currents was analyzed. PYR1 in the absence of ABA had a very small effect on the observed inward currents (Fig. 1.4). However, when ABA was injected into oocytes expressing these same genes, the inward currents were dramatically increased (Fig. 1.4). The inward currents of SLAC1, OST1, ABI1, and PYR1 in the presence or absence of ABA were decreased over time, characteristic of SLAC1 currents in *Xenopus* oocytes (Fig. 1.5).
A. SLAC1-Yc + OST1-Yn or CPK23-Yn

B. SLAC1-Yc + OST1-Yn or CPK23-Yn at -180 mV

Figure 1.4. (A) SLAC1 and regulating kinases were bound to split-YFP constructs to force a stronger interaction in Xenopus oocytes. In the presence of split-YFP, the SLAC1/OST1 interaction was strongly enhanced, while the SLAC1/CPK23 was approximately the same. Both SLAC1/OST1 and SLAC1/CPK23 demonstrated inhibition of inward currents in the presence of ABI1. The SLAC1 and OST1 system in the presence of ABI1, PYR1, and ABA demonstrated large inward currents, almost the same size as SLAC1 and OST1 in the absence of ABI1. (B) This is apparent at the -180 mV voltage level.
SLAC1-Yc + OST1-Yn + ABI1 + PYR1

A. No ABA

B. + ABA

Figure 1.5. Raw current data for SLAC1-Yc + OST1-Yn + ABI1 + PYR1 either (A) in the absence of ABA or (B) after injecting 500 µM ABA prior to recordings. Many of the oocytes showed large SLAC1 currents in the presence of ABA. The current is time-dependent and deactivated over time, characteristic of inward SLAC1 currents in *Xenopus* oocytes. Zero current levels are indicated by arrows on the left of the recorded current.
Mutation Effects on an ABA Signaling Pathway in *X. laevis* oocytes

Potential phosphorylation sites in the cytosolic loop regions of SLAC1, consisting of either serine or threonine amino acids, were mutated to either disable the phosphorylation site with alanine substitution, or to mimic a phosphorylation event with aspartate substitution. SLAC1 was expressed with OST1 in oocytes, using split YFP to enhance interaction of the two proteins. Among the potential phosphorylation sites, two showed a significant current inhibition when the alanine-mutated SLAC1 was compared with wild type. The SLAC1 amino acid threonine 437 and serine 442 both showed similar mutation effects in *Xenopus* oocytes. When the amino acid was mutated to alanine, the currents were reduced to approximately half of the wild type SLAC1 group (Fig. 1.6). When the same amino acids were mutated to aspartate, the currents were more similar to the wild type group, with a significant difference when compared to the alanine mutation of the same amino acid (Fig. 1.6). The inward currents in all of the mutant SLAC1 groups were deactivated over time, similar to the SLAC1 + OST1 control, suggesting that SLAC1 is at least partially active when the potential phosphorylation sites were mutated to alanine (Fig. 1.7).
Figure 1.6. (A) Two possible phosphorylation sites on SLAC1 were found to have an effect on measured inward anion currents. The alanine mutations displayed approximately half of the current of the wild type SLAC1, while the aspartate mutations displayed a current level more similar to wild type, especially at the -100 mV voltage level. (B) At -180 mV, the differences in current magnitudes are maximized.
Figure 1.7. (A) The time-dependent SLAC1 currents are visible in the presence of kinase OST1. (B) When threonine 437 of SLAC1 was mutated to alanine, the inward currents were significantly inhibited. When the same amino acid was mutated to aspartate, the inward currents were similar to control SLAC1. (C) When serine 442 of SLAC1 was mutated to alanine, the inward currents were also significantly inhibited. When mutated to aspartate, the currents were more similar to control SLAC1.
Two previously studied (Miyazono et al. 2009) point mutation sites of ABI1 were analyzed in *Xenopus* oocytes. The mutation ABI1 D413L is believed to disable the phosphatase activity of ABI1 (Miyazono et al. 2009). This is consistent with the observed effect in oocytes, since ABI1 D413L did not eliminate SLAC1 currents as wild type ABI1 did (Figs. 1.4 and 1.8). The other point mutation studied, ABI1 W300L, is believed to prevent the interaction of ABI1 with ABA receptors, such as PYR1 (Miyazono et al. 2009). In oocyte experiments, W300L was able to inhibit SLAC1 currents, exhibiting behavior similar to wild type ABI1. However, PYR1 in the presence of ABA was not able to reestablish large SLAC1 currents, as would be possible using wild type ABI1 (Figs. 1.4 and 1.8), suggesting an impaired interaction of ABI1 W300L with PYR1.
Figure 1.8. (A) Two point mutations in the phosphatase ABI1 were found to have different effects on SLAC1 currents in *Xenopus* oocytes. ABI1 D413L displayed an inactive ABI1 phenotype, in which ABI1 was not able to inhibit SLAC1-related inward currents. ABI1 W300L was able to inhibit SLAC1 currents similar to wild type ABI1, but demonstrated an inhibited response to PYR1 in the presence of ABA. (B) At -180 mV, the differences in current magnitudes are maximized.
1.5 Discussion:

Many compounds including ABA, CO$_2$, reactive oxygen species (ROS), nitric oxide (NO), and calcium are involved in the regulation of stomatal conductance (MacRobbie 1998; Schroeder et al. 2001; Klüsener et al. 2002; Hetherington and Woodward 2003). SLAC1 from guard cells, the first identified plant membrane anion channel gene, is crucial for ABA-dependent stomatal closure (Vahisalu et al. 2008). When SLAC1 was knocked out in Arabidopsis, low temperature phenotypes were observed, due to impaired stomatal closure in response to CO$_2$, ABA, and light stresses (Negi et al. 2008; Vahisalu et al. 2008). Knockouts also demonstrated the significant roles of SLAC1 in organic/inorganic ion homeostasis in Arabidopsis protoplasts (Negi et al. 2008). *Xenopus* oocyte permeability experiments showed that SLAC1 is permeable to Cl$^-$ and NO$_3^-$, but not HCO$_3^-$, SO$_4^{2-}$, or malate (Geiger et al. 2009; Lee et al. 2009). GUS staining and GFP fusion experiments demonstrated that SLAC1 was localized to the plasma membrane of guard cells in planta (Negi et al. 2008; Vahisalu et al. 2008). In *slac1* mutant plants, calcium and ABA activated anion channels were inhibited in guard cells (Vahisalu et al. 2008). SLAC1 is believed to have a five-helix transmembrane pore with a highly conserved phenylalanine residue that serves as a gate for the channel (Chen et al. 2010). Mutation of this residue into one with a small, nonpolar side chain allows SLAC1 to be active in the absence of regulating
kinases, though the ion selectivity of the channel is not altered (Chen et al. 2010). However, mutation of this tryptophan gate into the polar amino acid lysine blocked kinase activity and inhibited SLAC1 activity in Xenopus oocytes (Chen et al. 2010).

Recent experimentation showed that SLAC1 is activated by kinases CPK23 and OST1, as well as CPK21 and CPK6 to a smaller degree. Arabidopsis cpk23 and ost1 mutant plants displayed hindered ABA activation of anion channels in guard cells (Geiger et al. 2009; Geiger et al. 2010). In vitro kinase assay experiments showed that CPK23 and OST1 phosphorylate the N-terminus of SLAC1 (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010). Some SLAC1 N-terminal phosphorylation sites involved with OST1 have recently been identified (Vahisalu et al. 2010). Fluorescence imaging with split YFP in Xenopus oocytes, Arabidopsis mesophyll protoplasts, and Nicotiana benthamiana epidermal cells revealed that SLAC1 interacts with CPK23 and OST1 (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010; Vahisalu et al. 2010). The phosphatase ABI1 was also shown to interact with these two kinases (Geiger et al. 2009; Geiger et al. 2010). Xenopus oocyte electrophysiology demonstrated that SLAC1 is activated in the presence of CPK23 or OST1, and that PP2Cs ABI1, ABI2, and PP2CA are capable of inhibiting the kinase-regulated SLAC1 channel activity (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010).
An ABA-Dependent Signaling Pathway in *Xenopus* Oocytes

Though some variability was observed, SLAC1 alone is believed to exhibit little to no channel activity in *Xenopus* oocytes (Lee et al. 2009; Geiger et al. 2009; Geiger et al. 2010), and this is consistent with my results (Fig. 1.1). However, SLAC1 functions as an anion channel in the presence of protein kinases OST1 (Lee et al. 2009; Geiger et al. 2009) and CPK23 (Geiger et al. 2010). In my research, SLAC1 was significantly activated by both CPK23 and OST1, though SLAC1 was not activated by their respective homologues, CPK31 and SnRK2.3 (Fig. 1.1), as was noted in recent literature (Geiger et al. 2009; Geiger et al. 2010). The PP2C ABI1 very strongly inhibited the SLAC1 currents (Fig. 1.2), as expected, but the ABA receptor PYR1 was not effective in causing ABA regulation of SLAC1 in these studies (Geiger et al. 2009; Geiger et al. 2010). In my research at first, 50 µM ABA was added to the oocyte incubation solution and recording bath solution in an attempt to activate PYR1 and inhibit ABI1 activity, but electrophysiology experiments showed that any differences between groups with and without PYR1 were not significant (data not shown). Next, 500 µM ABA was injected into oocytes prior to recording, and a slight reactivation of the anion channel was observed (Fig. 1.3).
Recent literature noted that using split YFP in *Xenopus* oocytes resulted in enhanced interaction between potential channels and particular regulators (Geiger et al. 2009). Therefore I attached SLAC1 and the kinase of interest to the C- and N-terminal parts of split YFP to enhance interactions. Electrophysiology revealed that SLAC1 and OST1 resulted in much larger inward currents with fused split YFP (Fig. 1.4) and that ABI1 still inhibited the currents effectively (Fig. 1.4). Interestingly, in the presence of PYR1 with injected ABA, the channel activity was almost completely restored (Fig. 1.4 and 1.5), unlike the same system without split YFP (Fig. 1.3). This suggests that weak interaction of SLAC1 with the kinase of interest is the reason why PYR1-mediated inhibition of ABI1 was not previously observed.

At least one serine residue close to the N-terminus, amino acid 120, of SLAC1 is believed to be phosphorylated by OST1, and some others are crucial for channel function (Vahisalu et al. 2010). To locate new sites of interaction between SLAC1 and OST1, potential phosphorylation sites in the cytosolic loop regions of SLAC1 were mutated into alanine or aspartate. Threonine 437 and serine 422 are both suspected of being phosphorylation sites involved in SLAC1 activation, since alanine mutations inhibited SLAC1 currents, and aspartate mutations displayed SLAC1 currents more similar in size to wild type SLAC1 (Figs. 1.6 and 1.7).
A recent study used the crystal structures of ABA + PYL1 and the ABA + PYL1 + ABI1 complex to analyze the interaction of PYL1 with ABI1 in the presence of ABA (Miyazono et al. 2009). Among the observed structural properties was tryptophan 300 of ABI1, a side chain that interacts with the hydrophobic pocket of ABA-bound PYL1, and aspartate 413 of ABI1, an active-site residue that is highly conserved among PP2C phosphatases (Miyazono et al. 2009). To test these effects, both amino acids were mutated to lysine, and the ABA signaling pathway was reconstituted in *Xenopus* oocytes. ABI1 D413L was found to be inactive in this system, since the mutated ABI1 did not inhibit SLAC1 + OST1 currents as wild type ABI1 would (Fig. 1.8). ABI1 W300L, on the other hand, inhibited SLAC1 + OST1 currents, but was not hindered by ABA-bound PYR1, likely due to lack of binding between ABI1 and the PYR1 + ABA complex (Fig. 1.8).

Our findings demonstrate that *Xenopus* oocytes can be useful tools for the functional analysis of signaling pathways. Further research for potential components of the ABA-dependent signaling pathway, structural analyses of the constituents involved, and interaction studies are necessary for the understanding of environmentally influenced and ABA mediated stomatal regulation.

1.6 References:


MacRobbie EAC (2000) ABA activates multiple Ca\textsuperscript{2+} fluxes in stomatal guard cells, triggering vacuolar K\textsuperscript{+} (Rb\textsuperscript{+}) release. *Proc. Natl. Acad. Sci. USA* 97:12361-12368


II.

$K^+$ selective transport by the OsHKT2;4 transporter from rice (*Oryza sativa*) with atypical $Na^+$ transport properties and competition in permeation of $K^+$ over $Mg^{2+}$ and $Ca^{2+}$ ions
2.1 Abstract:

Members of the class II of HKT transporters, which have thus far only been isolated from grasses, were found to mediate Na\(^+\)-K\(^+\) co-transport and at high Na\(^+\) concentrations preferred Na\(^+\)-selective transport, depending on the ionic conditions. Class I of HKT transporters has been shown to play a central role in salinity tolerance of plants. But the physiological functions of the class II K\(^+\) transporting type of HKT transporters remain unknown. The genetically tractable rice (background Nipponbare) possesses two predicted K\(^+\) transporting class II HKT transporter genes, OsHKT2;3 and OsHKT2;4. In this study, we have characterized the ion selectivity of the class II rice (*Oryza sativa*) HKT transporter, OsHKT2;4, in yeast and *Xenopus laevis* oocytes. OsHKT2;4 rescued the growth defect of a K\(^+\) uptake deficient yeast mutant. GFP-OsHKT2;4 is targeted to the plasma membrane in transgenic plant cells. OsHKT2;4-expressing oocytes exhibited strong K\(^+\) permeability. Interestingly, however, K\(^+\) influx in OsHKT2;4-expressing oocytes did not require stimulation
by extracellular Na\(^+\), in contrast to other class II HKT transporters. Furthermore, OsHKT2;4-mediated currents exhibited permeabilities to both Mg\(^{2+}\) and Ca\(^{2+}\), in the absence of competing K\(^+\) ions. Comparative analyses of Ca\(^{2+}\) and Mg\(^{2+}\) permeabilities in several HKT transporters, including AtHKT1;1, TaHKT2;1, OsHKT2;1, OsHKT2;2 and OsHKT2;4 revealed that only OsHKT2;4 and to a lesser degree TaHKT2;1 mediate Mg\(^{2+}\) transport. TaHKT2;1 did not exhibit a large Ca\(^{2+}\) permeability. However, cation competition analyses demonstrate that the selectivity of both of these class II HKT transporters, OsHTK2;4 and TaHKT2;1, for K\(^+\) is dominant over divalent cations, suggesting that Mg\(^{2+}\) and Ca\(^{2+}\) transport via OsHKT2;4 may be small and would depend on competing K\(^+\) concentrations in plants.

### 2.2 Introduction:

K\(^+\) homeostasis is vital for growth and development of glycophytic plants. K\(^+\) and Na\(^+\) are chemically similar and are generally present at similar concentrations in non-saline soils. However, high concentrations of Na\(^+\) over 100 mM can occur in arid/semi-arid environments and irrigated soils. Glycophytic plants selectively accumulate far more K\(^+\) than Na\(^+\) in the cytoplasm when the proportion of the two ions in the environment is similar (Flowers and Läuchli, 1983). K\(^+\) taken up by roots is distributed to leaves. K\(^+\) ions have diverse indispensable functions in plant cells including
osmoregulation, cell expansion, enzyme activation, protein synthesis, 
membrane polarization and photosynthesis (Glass, 1983; Schroeder et al., 
1994; Véry and Sentenac, 2003; Gierth et al., 2005). \( K^+ \) deficiency causes 
several deleterious effects including a decrease in cytosolic pH (Walker et al., 
1996; Walker et al., 1998), resulting in substantial reductions in growth 
accompanied by damage to mature leaves and death of meristems (Flowers 
and Läuchli, 1983; Gierth and Mäser, 2007).

Multiple \( K^+ \) transport pathways across membranes contribute to the 
uptake and distribution of \( K^+ \) in plants, and many genes encoding channels 
and transporters that are permeable to \( K^+ \) have been identified in plants 
(Schroeder et al., 1994; Kwak et al., 2001; Lacombe et al., 2001; Véry and 
Sentenac, 2003; Gierth and Mäser, 2007; Lebaudy et al., 2007; Ward et al., 
2009). The identified \( K^+ \) channels/transporters are presently classified into six 
families (Lebaudy et al., 2007).

Plant HKT transporters have mainly been characterized as monovalent 
cation transporters. The first characterized HKT transporter, TaHKT2;1, 
(Schachtman and Schroeder, 1994) from wheat was found to mediate \( Na^+\)-
coupled \( K^+ \) transport and \( Na^+ \) transport at high \( Na^+ \) concentrations that occur 
under salinity stress conditions (Rubio et al., 1995; Gassmann et al., 1996; 
Laurie et al., 2002). HKT transporters identified from many different plant 
species to date can be divided into 2 subgroups, class I and class II, based on 
phylogenetic analyses (Mäser et al., 2002; Platten et al., 2006; Horie et al.,
Class I HKT transporters, including AtHKT1;1 from *Arabidopsis*, shows a more Na\textsuperscript{+} selective transport activity (Uozumi et al., 2000; Horie et al., 2001; Mäser et al., 2002; Ren et al., 2005; Horie et al., 2009; Hauser and Horie, 2010). In contrast, class II HKT transporters including TaHKT2;1 show robust K\textsuperscript{+} permeability. In recent years, most attention has focused on the Na\textsuperscript{+} transporting HKT transporter properties and functions due to: (i) their unique Na\textsuperscript{+} channel-like transport properties (Rubio et al., 1995; Gassmann et al., 1996; Liu et al., 2000; Horie et al., 2001; Ren et al., 2005; Corratgé et al., 2007; Jabnoune et al., 2009); (ii) the presence of class I HKT transporters with large Na\textsuperscript{+} permeabilities (Uozumi et al., 2000; Horie et al., 2001; Jabnoune et al., 2009; Yao et al., 2010) and (iii) the central relevance of class I HKT Na\textsuperscript{+} transporters for mediating salinity resistance in plants (Mäser et al., 2002a; Berthomieu et al., 2003; Ren et al., 2005; Sunarpi et al., 2005; Horie et al., 2006; Huang et al., 2006; Rus et al., 2006; Byrt et al., 2007; Davenport et al., 2007; Horie et al., 2009; Møller et al., 2009).

HKT transporters have been proposed to include four selectivity filter-pore-forming (“P-loop”) domains, each sandwiched by two transmembrane domains, which are distantly homologous to the bacterial K\textsuperscript{+} channel KcsA (Durell and Guy, 1999; Durell et al., 1999; Mäser et al., 2002). Furthermore, a
glycine residue at the filter position in each P-loop, which corresponds to the first glycine of “the GYG motif” that is highly conserved among K⁺ channels (Uozumi et al., 1995; Doyle et al., 1998), was found in HKT transporters (Durell and Guy, 1999; Mäser et al., 2002; Hauser and Horie, 2010). Class I HKT transporters, however, were found to have a serine residue instead of this glycine residue at the filter position of the first P-loop region in contrast to class II HKT transporters retaining all four glycines at the four filter positions (Hauser and Horie, 2010). OsHKT2;1 from rice is a unique class II transporter that exhibits features of class I transporters such as conservation of this serine residue at the filter position of the first P-loop and poor K⁺ permeability (Horie et al., 2001; Mäser et al., 2002; García-de Blas et al., 2003; Horie et al., 2007; Yao et al., 2010). Note that a non-selective alkali cation permeability and K⁺ permeability of OsHKT2;1, was characterized using *Xenopus laevis* oocytes, in other research (Golldack et al., 2002). Biophysical transport analyses using *X. laevis* oocytes and yeast, expressing chimeric fusions and point-mutated DNA constructs of class I and class II HKT transporters, TaHKT2;1, AtHKT1;1, OsHKT2;1 and OsHKT2;2, demonstrated the contribution of the glycine residues in the four pore-loops to K⁺ permeability of class II HKT transporters (Mäser et al., 2002; Tholema et al., 2005).

Whereas important Na⁺-transporting functions of HKT transporters with a serine residue in the first P-loop have been identified *in planta* (Mäser et al., 2002a; Berthomieu et al., 2003; Sunarpi et al., 2005; Horie et al., 2006; Horie
et al., 2007; Møller et al., 2009), the in planta function of the four glycine containing class II HKT transporters remain unknown. Multiple HKT genes were found in the japonica rice cultivar Nipponbare (García-de Blás et al., 2003). Seven full length OsHKT transporter genes were identified, consisting of four class I transporters and three class II transporters including OsHKT2;1 (Horie et al., 2001; García-de Blás et al., 2003; Platten et al., 2006). OsHKT2;3 and OsHKT2;4 are the most closely related among all analyzed HKT transporters, and share approximately 93% identity at the amino acid sequence level. OsHKT2;3 and OsHKT2;4 are the only class II transporters that conserve glycines at the four pore-loop filter positions in Nipponbare. An additional OsHKT2;2 gene encoding a K⁺-permeable class II transporter was isolated from a salt tolerant indica cultivar, Pokkali (Horie et al., 2001) and expression in plant cells exhibited Na⁺-coupled K⁺ transport (Yao et al., 2010), confirming previous cation selectivity studies in yeast and Xenopus oocytes (Horie et al., 2001). OsHKT2;2 is a non-full length pseudogene in Nipponbare (García-de Blás et al., 2003). Thus OsHKT2;3 and OsHKT2;4 provide the only class II HKT transporters in the genetically tractable Nipponbare rice background. However, first their cation selectivity properties need to be analyzed.

In the present study we have analyzed the ion selectivity of OsHKT2;4 using yeast and X. laevis oocytes. We show that OsHKT2;4 is targeted to the plasma membrane in transgenic plant cells and that OsHKT2;4 exhibits a
robust K⁺ permeability in both expression systems. Interestingly, however, OsHKT2;4 did not show Na⁺-coupled K⁺ transport unlike the case of other class II HKT transporters and OsHKT2;4-mediated K⁺ transport is shown here to be Na⁺ independent. Moreover, OsHKT2;4 exhibits a smaller Na⁺ permeability compared to TaHKT2;1 and the other HKT transporters analyzed in the present study. OsHKT2;4 expressed in X. laevis oocytes has recently been found to exhibit permeability to a wide range of cations including divalent cations such as Ca²⁺ and Mg²⁺ (Lan et al., 2010). Here, independent analysis of Ca²⁺ and Mg²⁺ permeabilities among five plant HKTs: AtHKT1;1, TaHKT2;1, OsHKT2;1, OsHKT2;2 and OsHKT2;4, in X. laevis oocytes reveal that TaHKT2;1 shows a small Mg²⁺ permeability. In contrast, OsHKT2;4 exhibits permeability for both Mg²⁺ and Ca²⁺. Competition experiments however show, that both OsHKT2;4 and TaHKT2;1 exhibit stronger selectivity for the monovalent cation K⁺, which out-competes Ca²⁺ and Mg²⁺ transport.

2.3 Materials and Methods:

Expression of OsHKTs in Yeast.

All OsHKT cDNAs were subcloned downstream of the Gal1 promoter in the plasmid pYES2 (Invitrogen). The K⁺ uptake deficient CY162 [MATa, Δtrak1, trk2::pCK64, his3, leu2, ura3, trp1, ade2] (Anderson et al., 1992) and the Na⁺ sensitive G19 [MATa, his3, leu2, ura3, trp1, ade2, ena1::HIS3::ena4] (Quintero
et al., 1996) *Saccharomyces cerevisiae* strains were used. Selection of transformants and subsequent growth assays using an arginine phosphate (AP) medium (Rodríguez-Navarro and Ramos, 1984) were performed as described previously (Rubio et al., 1999; Horie et al., 2001). Briefly, for both complementation and Na\(^+\) sensitivity analyses, AP media supplemented with 0.8 g/liter CSM-ura (Bio101), 2% (w/v) galactose, 0.6% (w/v) sucrose, 2% (w/v) agar, and the indicated concentrations of KCl and NaCl were used. Three independent clones per construct were analyzed under all conditions and plates were incubated at 30°C for 4 days (complementation) or 10 days (Na\(^+\) sensitivity).

**OsHKT expression constructs and electrophysiology in *X. laevis* oocytes**

For the synthesis of *TaHKT2;1, OsHKT2;1* and *OsHKT2;2* cRNA, previously reported constructs were used (Schachtman and Schroeder, 1994; Horie et al., 2001). *OsHKT2;3, OsHKT2;4* and *AtHKT1;1* cDNAs were subcloned into the pXβG-ev1 vector (Preston et al., 1992). Each cRNA was transcribed from linearized plasmid constructs using the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion).

Oocytes were kept for 1 to 3 d at 18°C in either a ND-96 solution or a modified Barth’s solution. Approximately 50 ng of cRNA, in a total volume of 50 nl, was injected into each *Xenopus laevis* oocyte for voltage clamp recordings. Recordings were performed 1-3 days after injection.
For the experiments shown in figures 2.3 to 2.5, voltage clamp recordings were performed using a dual-electrode voltage clamp amplifier (Nihon Kohden). Oocytes were perfused with a solution containing 6 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM MES-BTP, pH 5.5, 180 mM D-mannitol, and the indicated concentrations of Na- and K-Glutamate or 10 mM alkali cations as chloride salts. The ionic strength was kept constant by adding Tris-Glutamate. Lab-Trax-4/16 (World Precision Instruments) was used for electrophysiological measurements and voltage steps were applied from 0 to -150 mV in -15 mV decrements with a holding potential of -40 mV as described previously (Yao et al., 2010). A 3 M KCl agar bridge was used as a bath electrode.

For experiments shown in figures 2.6 to 2.8, recordings were performed with a Cornerstone (Dagan; Minneapolis, MN) TEV-200 two-electrode voltage clamp amplifier. Data analyses were performed using an Axon Instruments Digidata 1440A Low-Noise Data Acquisition System (Molecular Devices; Sunnyvale, CA). Oocytes were subjected to voltage pulses to record time-dependent currents (Schroeder, 1989) and to voltage ramps, with a holding potential of -40 mV. Data were low-pass filtered at 50 Hz throughout all recordings. Oocytes were bathed in 10 mM Tris, 0.3 mM EGTA (except in solutions containing calcium). The indicated concentrations of MgCl$_2$, CaCl$_2$, NaCl, and KCl, were added at pH = 7.4 (with Tris base, glutamic acid, or HCl) and osmolalities were adjusted to 220-260 mosmol/kg with D-mannitol. Oocytes were impaled with electrodes filled with 3 M KCl. Error bars
indicate standard error of the mean. All experiments were performed at room temperature.

Relative permeability ratios were calculated as previously reported (Fatt and Ginsborg, 1958; Schmidt and Schroeder, 1994). Experiments were analyzed in environments in which Ca$^{2+}$ or Mg$^{2+}$ were the only permeable cations in the bath solution and the intracellular K$^{+}$ concentration was valued at 100 mM (Schroeder et al., 1994).

**Mesophyll protoplast transformation and confocal microscopy**

Arabidopsis mesophyll protoplasts were transformed following a published procedure (Sheen, 2002). Protoplasts were incubated at 20˚C in the dark for at least 16 hours before microscopy analysis. For FM4-64 protoplast staining, the dye was directly added to the suspension at a final concentration of 17 µM (Horie et al., 2007). Fluorescence microscopy analyses were carried out after five minutes of incubation.

Confocal microscope analyses were performed using a Nikon PCM2000 (Bio-Rad, Germany) laser scanning confocal imaging system. For GFP detection, excitation was at 488 nm and detection between 515 and 530 nm. For the chlorophyll and FM4-64 detection, excitation was at 488 nm and detection over 570 nm. The images acquired from the confocal microscope were processed using ImageJ (http://rsbweb.nih.gov/ij/).
2.4 Results:

Functional expression of OsHKT2;3 and OsHKT2;4 in yeast

The OsHKT2;3 and OsHKT2;4 transporters retain the four selectivity
filter Gly residues, typical of class II HKT transporters (Garcia-de-Blas et al.,
2003; Horie et al., 2009; Hauser and Horie, 2010). We therefore expressed
OsHKT2;3 and OsHKT2;4 in a high-affinity K⁺-uptake-deficient mutant of yeast
strain, CY162 (Anderson et al., 1992), and performed growth analyses under
K⁺-limited conditions. All transformants showed no remarkable difference in
their growth when K⁺ was supplied at a high 10 mM concentration (Fig. 2.1A).
However, only the expression of OsHKT2;4 and OsHKT2;2 could rescue the
growth defect of the mutant in the presence of 0.1 mM KCl (Fig. 2.1B).
OsHKT2;3, which shows more than 93% identity to OsHKT2;4 at the amino
acid sequence level, did not complement the mutations (Fig. 2.1B).
Figure 2.1. OsHKT2;4 complements high-affinity K$^+$ uptake deficient mutant *S. cerevisiae* strain CY162. CY162 cells were transformed with an empty vector pYES2 and members of the *Oryza Sativa* HKT family, OsHKT2;1, OsHKT2;2, OsHKT2;3 and OsHKT2;4. Growth was monitored on arginine phosphate (AP) medium. (A) Growth of each CY162 transformant on AP medium supplemented with 10 mM KCl, incubated at 30°C for 2 days. (B) Growth of each CY162 transformant on AP medium supplemented with 0.1 mM KCl, incubated at 30°C for 4 days. Three independent clones were tested for every condition with similar results.
The TaHKT2;1 transporter from wheat has been shown to exhibit low-affinity Na⁺ transport with Na⁺-channel-like transport properties in the presence of high Na⁺ concentrations (Rubio et al., 1995; Gassmann et al., 1996), and similar Na⁺ transport properties were found for OsHKT2;1 and OsHKT2;2 (Horie et al., 2001; Yao et al., 2010). Therefore, we next expressed OsHKT proteins in a Na⁺ hypersensitive mutant yeast strain, G19 (Rubio et al., 1999). Increasing the concentration of NaCl in the growth medium caused severe growth defects in OsHKT2;1-expressing cells compared with vector-harboring control cells as previously reported (Horie et al., 2001) (Fig. 2.2A-D). In comparison, G19 cells expressing the K⁺-permeable transporters, OsHKT2;2 and OsHKT2;4, were able to grow on medium containing 200 mM NaCl although OsHKT2;2-expressing cells exhibit remarkably greater sensitivity to NaCl stress. In contrast, control cells no longer survived (Fig. 2.2D). OsHKT2;3-harboring cells exhibited very similar Na⁺ sensitivity to control cells (Fig. 2.2A-D).
Figure 2.2. OsHKT2;4 reduces salt sensitivity in growth inhibition tests using the Na⁺ hypersensitive mutant strain of S. cerevisiae, G19 in which all four ENA Na⁺ ATPases were deleted (MATα, his3, ura3, trp1, ade2, and ena1::HIS3::ena4). G19 yeast cells were transformed with an empty vector pYES2 and the indicated OsHKT transporters described in Figure 1. Growth was monitored on arginine phosphate (AP) medium. (A) Growth of each G19 transformant on AP medium supplemented with 1 mM KCl and either (A) 50 mM NaCl; (B) 100 mM NaCl; (C) 150 mM NaCl; or (D) 200 mM NaCl. Expression of the K⁺-uptake-mediating OsHKT2;4 (Fig. 1) and OsHKT2;2 transporters reduced salt sensitivity. As expected, the Na⁺ influx transporter OsHKT2;1 enhanced Na⁺ sensitivity under all conditions. G19 cells were grown at 30°C for 10 days. Three independent clones were tested for every condition with similar results.
Monovalent alkali cation selectivity of OsHKT2;4 in *Xenopus laevis* oocytes

To investigate ion selectivity properties of class II HKT transporters, two-electrode voltage clamp experiments using *X. laevis* oocytes were performed. OsHKT2;3- and OsHKT2;4-dependent currents were recorded by exposing each OsHKT2 cRNA-injected oocyte to bath solutions, supplemented with 10 mM alkali cation salts. In the case of OsHKT2;3 cRNA-injected oocytes, none of the alkali cations evoked significant currents that differed from water-injected control oocytes (data not shown). In contrast, however, OsHKT2;4 cRNA-injected oocytes showed large currents for every alkali cation tested (Fig. 2.3A). OsHKT2;4-mediated currents shared a strong inward rectification at voltages negative of -135 mV for all cations tested (Fig. 2.3). Among the five alkali cations, K\(^+\) showed the most positive reversal potential, followed by Rb\(^+\) \(\approx\) Cs\(^+\) and Na\(^+\) \(\approx\) Li\(^+\), suggesting a higher relative K\(^+\) permeability (Fig. 2.3). Control water-injected oocytes showed small background currents (Fig. 2.3). Given that HKT transporters exhibit characteristic Na\(^+\) uniport activity at high Na\(^+\) concentrations, amplitudes of OsHKT2;4-mediated inward currents were compared with those of OsHKT2;1 and OsHKT2;2 at voltages negative of -150 mV, recorded with 10 mM NaCl in the bath solution. The results revealed substantial differences in current amplitudes in response to 10 mM NaCl between OsHKT2;4 and the other two class II OsHKT transporters (Fig. 2.3B).
**Figure 2.3.** OsHKT2;4 expression in *X. laevis* oocytes mediates inward ion currents for all five alkali cations analyzed. (A) The current-voltage relationships from oocytes injected with 50 ng of *OsHKT2;4* cRNA are shown. Oocytes were bathed in solutions supplemented with 10 mM alkali monovalent cations as chloride salts. Note that only background currents of water-injected control oocytes bathed in a 10 mM NaCl solution are present as a representative control, as no significant differences were found among the five ionic conditions in controls. Currents were recorded from a holding potential of -40 mV using a step command with 15-mV decrements as described in Yao et al. (2010) (see also “Materials and Methods”). Error bars represent ± SE (n = 5 for water injected control and n = 6 for OsHKT2;4-expressing oocytes at each condition). (B) Amplitudes of OsHKT2-mediated inward currents, recorded at -150 mV. Voltage clamp experiments were performed in the presence of 10 mM NaCl. Note that data for OsHKT2;4-expressing and water-injected oocytes are the same as the recordings presented in A, and 12.5 ng of cRNA was injected into oocytes for the recordings of OsHKT2;1- and OsHKT2;2-mediated currents. Error bars represent ± SE (n = 5 for water injected control and n = 6 for OsHKT2-expressing oocytes).
The class II HKT transporters with four glycine residues in their selectivity filter domains characterized to date show Na\(^+\)-K\(^+\) co-transport (Yao et al., 2010). We thus analyzed combined effects of Na\(^+\) and K\(^+\) on OsHKT2;4-expressing oocytes to determine whether OsHKT2;4 can mediate similar Na\(^+\)-K\(^+\) co-transport. In the first type of experiments, OsHKT2;4-mediated currents were recorded in bath solutions where Na\(^+\) was set to 0.3 mM and the K\(^+\) concentration was increased from 0.3 mM to 10 mM. The current (I) - voltage (V) relationships of OsHKT2;4-expressing oocytes revealed significant positive shifts in the reversal potential and increases in inward currents as the K\(^+\) concentration of the bath solution was increased (Fig. 2.4A), exhibiting a clear K\(^+\) permeability. In contrast, the current-voltage relationship of OsHKT2;4-expressing oocytes bathed with constant 0.3 mM K\(^+\) with increasing Na\(^+\) concentrations exhibited only slight positive reversal potential shifts such that increases in the extracellular Na\(^+\) concentration from 0.3 mM to 3.0 mM and 10 mM led to approximately +9 mV and +5 mV reversal potential shifts, respectively, in comparison with 0.3 mM extracellular Na\(^+\) concentration (Fig. 2.4B). The transport properties of OsHKT2;4 showed marked differences to other class II plant HKT transporters characterized thus far which showed much larger and increasing positive shifts in the reversal potential upon in increasing extracellular Na\(^+\) concentration (Rubio et al., 1995; Gassmann et al., 1996; Horie et al., 2001; Yao et al., 2010).
Figure 2.4. OsHKT2;4 exhibits robust K\(^+\) but weak Na\(^+\) permeability. Current-voltage relationships from oocytes injected with 50 ng of OsHKT2;4 cRNA are shown. (A) OsHKT2;4-expressing oocytes were bathed in a 0.3 mM Na\(^+\) solution supplemented with the indicated concentrations of K\(^+\). (B) OsHKT2;4-expressing oocytes were bathed in a 0.3 mM K\(^+\) solution supplemented with the indicated concentrations of Na\(^+\). (A-B) Currents were recorded from a holding voltage of -40 mV using step commands with 15-mV decrements. Note that only the background currents of water-injected control oocytes bathed in a 0.3 mM Na\(^+\) & 10 mM K\(^+\) solution (A) or a 0.3 mM K\(^+\) & 10 mM Na\(^+\) solution (B) were presented as representative controls as no significant difference was found among the ionic conditions tested. Error bars represent ± SE (n = 5 for water injected control and n = 12-13 for OsHKT2;4-expressing oocytes at each condition). K\(^+\) and Na\(^+\) were added to the bath solutions as glutamate salts.
We further analyzed OsHKT2;4-mediated K\textsuperscript{+} currents by exposing OsHKT2;4-expressing oocytes to increasing extracellular K\textsuperscript{+} concentrations with no added extracellular Na\textsuperscript{+}. Increasing the K\textsuperscript{+} concentration from 0.3 mM to 10 mM resulted in significant positive shifts in the reversal potential (Fig. 2.5). Reversal potentials shifted by approximately +18 mV when the extracellular K\textsuperscript{+} concentration was increased from 0.3 mM to 3 mM (Fig. 2.5). These data provide clear evidence that OsHKT2;4 transports K\textsuperscript{+} and is not an obligate Na\textsuperscript{+}-K\textsuperscript{+} co-transporter.
Figure 2.5. OsHKT2;4 mediates inward $K^+$ currents in the absence of extracellularly added $Na^+$ in *X. laevis* oocytes. Current-voltage relationships from oocytes injected with 50 ng of *OsHKT2;4* cRNA are shown. During OsHKT2;4-mediated current recordings, the $K^+$ concentration of the bath solution was increased from 0.3 mM to 10 mM. Note that only the background currents of water-injected control oocytes bathed in a 10 mM $K^+$ solution were presented as a representative control as no significant difference was found among the three ionic conditions. Currents were recorded from a holding voltage of -40 mV using step commands with 15-mV decrements and error bars represent ± SE (n = 6 for each condition). $K^+$ was added to the bath solutions as a glutamate salt.
Divalent cation selectivity of HKT transporters in *Xenopus laevis* oocytes

During the above experiments, we noticed unusual shifts in the reversal potentials of OsHKT2;4-mediated currents, including when MgCl$_2$ and Mg-Glutamate concentrations were changed. Recently, OsHKT2;4 was independently reported to be a cation transporter showing substrate specificity to a wide range of cations including Ca$^{2+}$ and Mg$^{2+}$ (Lan et al., 2010). Based on our initial observations and this recent report, we next analyzed several members of the HKT family for Mg$^{2+}$ and Ca$^{2+}$ transport properties (Fig. 2.6).

Each HKT protein was exposed to 5 mM and high 50 mM concentrations of Mg$^{2+}$ and Ca$^{2+}$, as chloride salts, in the bath solution. The same conditions were also applied to water-injected oocytes as a control. As no significant differences in the resulting inward currents of water-injected control oocytes were observed, all control oocytes were combined into one group for simplicity (Fig. 2.6). Electrophysiological recordings were performed using both voltage pulse and voltage ramp protocols with similar findings, and the voltage ramp recordings were used to generate the current-voltage graphs in figure 2.6 with representative voltage pulse response time-dependent recordings shown in figure 2.7.

For the AtHKT1;1, OsHKT2;1, and OsHKT2;2 transporters (Uozumi et al., 2000; Horie et al., 2001), no significant differences in the current magnitudes were observed when the extracellular Mg$^{2+}$ concentrations were increased from 5 mM to 50 mM (Fig. 2.6A-C; P ≥ 0.114 at -150 mV; Wilcoxon
Signed Rank Test). The shifts in reversal potentials upon these tenfold-increases in Mg$^{2+}$ concentration were +1 mV for AtHKT1;1, +4 mV for OsHKT2;1, and +13 mV for OsHKT2;2. Note that this +13 mV shift in reversal potential indicates a small Mg$^{2+}$ permeability of OsHKT2;2 (Fig. 2.6C).

OsHKT2;4 and TaHKT2;1, on the other hand, showed a more significant current increase and positive reversal potential shift upon increasing the extracellular Mg$^{2+}$ concentration (Fig. 2.6D-E). For OsHKT2;4-mediated currents, an average +33 mV reversal potential shift was observed when increasing the extracellular MgCl$_2$ concentration from 5 mM to 50 mM. Moreover, an average +45 mV shift in the reversal potential of OsHKT2;4-dependent currents was observed when increasing the extracellular concentration of CaCl$_2$ from 5 to 50 mM. These results suggest that OsHKT2;4 is permeable to Mg$^{2+}$ and Ca$^{2+}$. A +45 mV shift for a tenfold Ca$^{2+}$ concentration increase also suggests that in addition to Ca$^{2+}$ permeability, Ca$^{2+}$ itself modulates other ion conductances in oocytes, possibly in the form of activating endogenous Ca$^{2+}$-activated Cl$^-$ channels (Cao et al., 1995). For the wheat transporter TaHKT2;1, reversal potentials shifted less dramatically, exhibiting an average shift of +20 mV with the same tenfold increase in MgCl$_2$ concentration. Upon increasing the CaCl$_2$ concentration, however, the reversal potential shift was negligible with an average shift of -2 mV (Fig. 2.6E). These results indicate permeability to Mg$^{2+}$. The lack of a clear measured shift in reversal potential with Ca$^{2+}$ increases for TaHKT2;1 is consistent with previous
results from unpublished pilot studies (W. Gassmann and Schroeder, unpublished observations). Nevertheless larger average inward currents were observed at 50 mM CaCl$_2$, indicating a possible small effect of Ca$^{2+}$ on TaHKT2;1-dependent transport. The relative permeability ratios for OsHKT2;4 ($P_{Mg}/P_K$ of 0.0203 and $P_{Ca}/P_K$ of 0.0108) and TaHKT2;1 ($P_{Mg}/P_K$ of 0.0187) were determined (Fatt and Ginsborg, 1958) using reversal potentials of OsHKT2;4-mediated currents from figure 2.6, and assuming an intracellular K$^+$ concentration of 100 mM (Schroeder et al., 1994). These results indicate a much stronger permeability towards K$^+$ than to either of the divalent cations, for both transporters. Note however, that this does not rule out uptake of Ca$^{2+}$ or Mg$^{2+}$ via OsHKT2;4 down a relatively steep electrochemical gradient, and depends on the relative competition among permeating cations. Analyses of the time-dependence of OsHKT2;4- and TaHKT2;1-mediated currents in response to voltage pulses showed differences between the two transporters (Fig. 2.7). Increases in whole-cell currents in oocytes expressing TaHKT2;1 in the presence of high 50 mM CaCl$_2$ indicate a possible small Ca$^{2+}$ permeability of TaHKT2;1, and a possible contribution to currents by endogenous Ca$^{2+}$ activated Cl$^-$ channels in oocytes (Figs. 2.6E and 2.7). Furthermore, OsHKT2;4-mediated currents exhibited a time-dependent activation at more negative voltages, in contrast to TaHKT2;1-mediated currents.
Figure 2.6. Five HKT family transporters were tested for Mg$^{2+}$ and Ca$^{2+}$ permeability in the presence of 5 mM and 50 mM extracellular concentrations of MgCl$_2$ or CaCl$_2$. AtHKT1;1 and OsHKT2;1 (A,B) showed small changes in the reversal potentials of HKT-mediated currents, even upon extracellular exposure to high 50 mM concentrations of these divalent cations. OsHKT2;4 and TaHKT2;1 (D,E) exhibited notable shifts in the reversal potentials in response to 50 mM Mg$^{2+}$ concentrations. OsHKT2;4 showed a +33 mV reversal potential shift upon increasing MgCl$_2$ concentration from 5 to 50 mM, and a +45 mV shift for CaCl$_2$ upon increasing concentration from 5 to 50 mM. TaHKT2;1 showed a +20 mV shift for MgCl$_2$ after increasing the bath concentration from 5 to 50 mM, and a -2 mV shift for a 5 to 50 mM CaCl$_2$ increase. Currents were recorded from a holding voltage of -40 mV using a 2.5-second ramp protocol ranging from 0 mV to -150 mV. Error bars represent ± SE (n = 6-53, depending on condition and transporter tested).
Figure 2.7. OsHKT2;4 mediates Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport. The addition of 50 mM Ca\textsuperscript{2+} or Mg\textsuperscript{2+} resulted in large, time dependent inward cation currents for both (B) OsHKT2;4 and (C) TaHKT2;1 compared to controls (A). (A) Small currents were detected in water-injected control oocytes. A bath solution containing 50 mM CaCl\textsubscript{2} was used for the control recording shown. (B) Similar current magnitudes were observed for the 50 mM Ca\textsuperscript{2+} and Mg\textsuperscript{2+} groups in OsHKT2;4. Slow time-dependent activation was commonly observed in OsHKT2;4-injected oocytes during hyperpolarized voltages. (C) Currents recorded in TaHKT2;1-expressing oocytes at the indicated MgCl\textsubscript{2} and CaCl\textsubscript{2} concentrations. Unlike in OsHKT2;4, slow time-dependent deactivation was commonly observed in TaHKT2;1-injected oocytes during hyperpolarized voltages. Zero current levels are shown by arrows on the left of the recorded currents.
Previous studies on animal Ca$^{2+}$ channels have shown appearance of a large Na$^+$ permeability when extracellular Ca$^{2+}$ was removed (Almers et al., 1984; Hille, 1992). Therefore, Na$^+$ or K$^+$ competition experiments with Mg$^{2+}$ and Ca$^{2+}$ were performed to determine the preference in cation permeabilities of OsHKT2;4- and TaHKT2;1 for monovalent vs. divalent cations (Fig. 2.8). OsHKT2;4 was exposed to 10 mM K$^+$ while TaHKT2;1 was analyzed with 10 mM Na$^+$/3 mM K$^+$ for the competition experiments, as these conditions trigger cation currents in the respective HKT transporters (Figs. 2.3A and 2.5) (Rubio et al., 1995). For OsHKT2;4-mediated currents, the reversal potential shifted by +5 mV upon addition of 50 mM Mg$^{2+}$ to the bath solution. Similarly, the reversal potential was shifted by +4 mV upon addition of 50 mM Ca$^{2+}$ to the bath solution. The average current magnitudes in the presence of 10 mM K$^+$ plus 50 mM Mg$^{2+}$ or 50 mM Ca$^{2+}$ groups were slightly larger than 10 mM K$^+$ alone, and these increases were not statistically significant at -150 mV (Fig. 2.8A; P > 0.095; Wilcoxon Signed Rank Test). These results indicate that Mg$^{2+}$ and Ca$^{2+}$ were substantially less permeable under competing 10 mM K$^+$ conditions, despite the high (50 mM) divalent cation concentrations tested. A small residual permeation of Mg$^{2+}$ or Ca$^{2+}$ may however occur. When TaHKT2;1-expressing oocytes (Fig. 2.8B) were exposed to 50 mM added Mg$^{2+}$ in competition experiments, the reversal potential shifted by +6 mV. When 50 mM Ca$^{2+}$ was added to the bath solution, the reversal potential shifted +3 mV. The average current magnitudes in the presence of 10 mM
Na\(^+\)/3 mM K\(^+\) plus 50 mM Mg\(^{2+}\) or 50 mM Ca\(^{2+}\) groups were also slightly larger than 10 mM Na\(^+\)/3 mM K\(^+\) alone, and the increases were statistically significant at -150 mV (Fig. 2.8B; P < 0.005; Wilcoxon Signed Rank Test). Although the differences in current magnitudes under the described competition settings were found to be significant for TaHKT2;1 using a paired and ranked statistical test, they were not large. These findings together show a competitive inhibition of the ability of these HKT transporters to transport Ca\(^{2+}\) and Mg\(^{2+}\) in the presence of permeating K\(^+\) and Na\(^+\) cations, even when high 50 mM [Mg\(^{2+}\)] or [Ca\(^{2+}\)] were extracellularly added to the 10-13 mM total [K\(^+\)] and [Na\(^+\)].
Figure 2.8. Inhibition of strong Mg\(^{2+}\) and Ca\(^{2+}\) permeabilities in OsHKT2;4 and TaHKT2;1 was observed in the presence of competing K\(^+\) and Na\(^+\) ions. (A) OsHKT2;4 was analyzed for Mg\(^{2+}\) and Ca\(^{2+}\) permeabilities in the presence of 10 mM K\(^+\). (B) TaHKT2;1 was analyzed for Mg\(^{2+}\) and Ca\(^{2+}\) permeabilities in the presence of 10 mM Na\(^+\) and 3 mM K\(^+\). (A) OsHKT2;4 showed a +5 mV reversal potential shift upon adding 50 mM MgCl\(_2\) to the 10 mM KCl competition control solution. Addition of CaCl\(_2\) also resulted in a small average +4 mV reversal potential shift. (B) TaHKT2;1 exhibited a +6 mV reversal potential shift upon adding 50 mM MgCl\(_2\) to the 10 mM NaCl, 3 mM KCl control solution. Addition of 50 mM CaCl\(_2\) to the control solution resulted in a smaller +3 mV reversal potential shift. Currents were recorded from a holding voltage of -40 mV using step commands with 30-mV decrements and error bars represent ± SE (n = 12-19, depending on condition and transporter tested).
Subcellular localization of OsHKT2;4 in plant cells

To determine the membrane localization of the OsHKT2;4 protein in plant cells, we constructed OsHKT2;4 fused with EGFP at the N-terminus (EGFP-OsHKT2;4). Localization analyses were performed using protoplasts of Arabidopsis mesophyll cells. Figure 2.9A-C shows protoplasts expressing the EGFP-OsHKT2;4 protein. The GFP fluorescence signal was uniformly present at the periphery of the cell (Fig. 2.9A and C). A small fluorescence signal was also detected inside cells, which may reflect endoplasmic reticulum localization, where the protein is assembled (Fig. 2.9A). As controls, we transformed protoplasts with free EGFP which showed the typical cytoplasmic and nuclear localizations (Fig. 2.9D-F). To better determine whether OsHKT2;4 shows plasma membrane localization, we stained protoplasts expressing EGFP-OsHKT2;4 with the plasma membrane marker FM4-64 (Fig. 2.9G-I) (Bolte et al., 2004; Horie et al., 2007). The green and red fluorescence signals from EGFP and FM4-64 in protoplasts expressing the EGFP-OsHKT2;4 protein (Fig. 2.9G and H) uniformly overlapped when the two images were combined (Fig. 2.9I). In comparison, when the same staining was performed with protoplasts expressing the free EGFP, the clear overlap of the two signals was not observed (Fig. 2.9J-L). In the confocal plane shown in figure 2.9J, the EGFP signal appeared to also be present at the periphery of the cell. This was due to the presence of the large central vacuole, which pushed the cytoplasm towards the plasma membrane. Together, these results
provide evidence that EGFP-OsHKT2;4 localizes to the plasma membrane of Arabidopsis mesophyll protoplasts, suggesting plasma membrane localization of OsHKT2;4 in plant cells.
Figure 2.9. Subcellular localization of EGFP-OsHKT2;4 in *Arabidopsis* mesophyll protoplasts. EGFP-OsHKT2;4 protein was transiently expressed in protoplasts of *Arabidopsis* mesophyll cells under the control of the CaMV 35S promoter. Fluorescence was analyzed by confocal microscopy. (A) EGFP fluorescence (green) from the chimeric EGFP-OsHKT2;4 protein shows fluorescence at the periphery of the protoplast. (B) Chlorophyll auto fluorescence (red) of the same protoplast shown in (A). (C) Overlay image of (A) and (B). (D) Control EGFP fluorescence (green) from the free EGFP. (E) Chlorophyll auto fluorescence (red) of the same protoplast shown in (D). (F) Overlay image of (E) and (D). (G) Confocal EGFP fluorescence (green) image from a focal plane of an EGFP-OsHKT2;4-expressing cell. (H) FM4-64 fluorescence image of the same protoplast shown in (G). (I) Overlay image of (G) and (H). (J) Confocal EGFP fluorescence (green) image of an EGFP-expressing cell. (K) FM4-64 fluorescence image of the same protoplast shown in (J). (L) Overlay image of (J) and (K).
2.5 Discussion:

HKT transporters are one of the best characterized Na\(^+\) permeable membrane proteins in plants. TaHKT2;1 was isolated by complementation screening of a K\(^+\) starvation-treated wheat root library using a high-affinity K\(^+\) uptake deficient mutant of yeast (Schachtman and Schroeder, 1994). Initial *X. laevis* oocytes voltage clamp analyses showed that all alkali cations tested affected TaHKT2;1-mediated outward currents (Schachtman and Schroeder, 1994). Subsequent ion selectivity analyses of TaHKT2;1 expressed in *X. laevis* oocytes and yeast revealed that TaHKT2;1 mediates high-affinity Na\(^+\)-K\(^+\) co-transport and also exhibits Na\(^+\)-selective low-affinity channel-like Na\(^+\) transport in the presence of mM concentrations of Na\(^+\) (Rubio et al., 1995; Gassmann et al., 1996). Complementary DNAs encoding HKT transporter family members have been identified in many plant species and the monovalent cation transport properties of the encoded proteins have been characterized, mainly in heterologous expression systems (Gassmann et al., 1996; Rubio et al., 1999; Liu et al., 2000; Horie et al., 2001; Liu et al., 2001; Garciadeblás et al., 2003; Horie et al., 2009; Hauser and Horie, 2010), and in a few exceptions in plant cells (Laurie et al., 2002; Horie et al., 2007; Yao et al., 2010). These analyses have shown that class I HKT transporters and OsHKT2;1 preferentially show Na\(^+\) transport activity (Uozumi et al., 2000;
Horie et al., 2001; Mäser et al., 2002; García-de Blas et al., 2003; Jabnoune et al., 2009; Yao et al., 2010).

Phylogenetic analyses of the identified HKT proteins showed that HKT transporters can be divided into at least two subgroups: class I and class II transporters, which show preferred Na\(^+\) selective transport in class I transporters, and K\(^+\) transport as well as Na\(^+\) transport for class II HKT transporters (Mäser et al., 2002; Platten et al., 2006; Horie et al., 2009; Hauser and Horie, 2010). Molecular genetic research demonstrated that Na\(^+\) transport activity mediated by members of the class I HKT transporter plays a key role in Na\(^+\)-tolerance and in Na\(^+\) exclusion from leaves in *Arabidopsis*, rice, and wheat (Mäser et al., 2002; Berthomieu et al., 2003; Ren et al., 2005; Sunarpi et al., 2005; Horie et al., 2006; Huang et al., 2006; Rus et al., 2006; Byrt et al., 2007; Davenport et al., 2007; Møller et al., 2009). Furthermore, OsHKT2;1, a unique class II transporter that mediates more Na\(^+\) selective transport and lacks robust K\(^+\) permeability, unlike the other typical class II transporters, has been shown to compensate for K\(^+\) deficiency through nutritional Na\(^+\) absorption and distribution in rice plants (Horie et al., 2007).

Compared to class I HKT transporters, little information exists on the functions of K\(^+\) transporting class II HKT transporters *in planta*. Thus far class II HKT transporters have only been identified in grasses, including wheat, rice, and barley (Wang et al., 1998; Huang et al., 2008). In this study, we analyzed the cation selectivity properties of the OsHKT2;4 transporter, surprisingly
showing that OsHKT2;4 exhibits a distinct ion selectivity for K$^+$ and Na$^+$ in comparison with other HKT transporters. OsHKT2;4 mediates robust inward K$^+$ currents, even without addition of extracellular Na$^+$, unlike that of other class II HKT transporters (Figs. 2.3A, 2.4A, 2.5 and 2.8), implying that OsHKT2;4 plays a role in K$^+$ homeostasis as a K$^+$ transporter/channel rather than a Na$^+$-K$^+$ co-transporter.

**Substantial differences between class II HKT transporters**

The K$^+$-permeable class II HKT transporters were found to retain a glycine residue at each filter position in the four putative selectivity pore-forming regions that have been proposed to be derived from an ancestral K$^+$ channel in bacteria (Durell and Guy, 1999; Durell et al., 1999; Mäser et al., 2002; Tholema et al., 2005). Ion transport analyses of plant HKTs and of the bacterial HKT homolog, KtrAB K$^+$ transport system, from *Vibrio alginolyticus*, demonstrated that the glycines at the filter positions are important contributors to robust K$^+$ permeability of HKT transporters (Mäser et al., 2002; Tholema et al., 2005). OsHKT2;3 and OsHKT2;4 transporters are the only two full-length class II transporters found in the genetically tractable *japonica* rice cv. Nipponbare genome, in which all four glycines at the filter positions are conserved as other typical class II transporters. Moreover, OsHKT2;3 and OsHKT2;4 show more than 93% identity at the amino acid sequence level. The expression of OsHKT2;3 did not cause any difference in the growth
response of either CY162 or G19 mutant yeast cells compared to vector-
harboring control cells (Figs. 2.1 and 2.2). Furthermore, no significant
OsHKT2;3-mediated currents were observed in various cation solutions in
comparison with water-injected control oocytes in two electrode voltage clamp
experiments (data not shown). These results suggest that OsHKT2;3 does not
exert cation uptake activity, at least under the tested heterologous expression
conditions, for unknown reasons. Note that the sequences of expression
constructs in yeast and oocytes were carefully checked and found to possess
no errors. Transient expression of EGFP-OsHKT2;3 revealed a plasma
membrane localization of the chimeric protein in protoplasts of Arabidopsis
mesophyll cells (data not shown) as found in similar analyses using an EGFP-
OsHKT2;4 expression construct (Fig. 2.9). The expression of OsHKT2;4
triggered robust growth responses in yeast cells and elicited significant
currents in oocytes (Figs. 2.1 to 2.8).

**K⁺ uniport function of OsHKT2;4 in yeast and X. laevis oocytes**

The expression of OsHKT2;4 rescued growth defects of CY162 yeast
cells that are deficient in high-affinity K⁺ uptake (Fig. 2.1B), similar to other
typical class II HKT transporters such as TaHKT2;1 and OsHKT2;2
(Schachtman and Schroeder, 1994; Horie et al., 2001). Robust K⁺ permeability
was also found in oocytes expressing OsHKT2;4 (Figs. 2.3A, 2.4A and 2.5).
However, a remarkable distinction was found in the properties of Na⁺ transport
between OsHKT2;4 and HKT transporters characterized to date. OsHKT2;4-mediated Na\(^+\) transport activity at high extracellular Na\(^+\) concentrations appears to be smaller in comparison to that of OsHKT2;1 and OsHKT2;2 (Fig. 2.3B). Increasing Na\(^+\) concentrations in a 0.3 mM K\(^+\) bath solution caused only slight positive shifts in the reversal potential of OsHKT2;4-expressing oocytes (Fig. 2.4B). Furthermore, OsHKT2;4-expressing G19 yeast cells showed the lowest sensitivity to extracellular NaCl, even lower than vector-harboring control cells (Fig. 2.2). The increased salt tolerance of yeast cells expressing OsHKT2;4 may be due to an increased K\(^+\) accumulation via OsHKT2;4 (Fig. 2.2), based on previous studies of Na\(^+\) sensitivities of HKT-expressing yeast lines (Rubio et al., 1995; Rubio et al., 1999; Yenus et al., 2002). Taken together, the present findings show that OsHKT2;4 has a distinct K\(^+\)/Na\(^+\) selectivity compared to other class II HKT transporters with four glycine residues such that OsHKT2;4 mediates robust channel-like K\(^+\) uniport independent of Na\(^+\).

Recent voltage clamp analyses of OsHKT2;4-expressing oocytes have indicated that OsHKT2;4 mediates transport of various cations, including divalent cations such as Ca\(^{2+}\) and Mg\(^{2+}\) (Lan et al., 2010). Together with plasma membrane localization of OsHKT2;4 in root hair cells of rice cv. Nipponbare plants, a novel physiological role of OsHKT2;4 in Ca\(^{2+}\)-related biological processes other than K\(^+\)/Na\(^+\) homeostasis was implicated (Lan et al., 2010). Independent experiments in the present study in which extracellular
MgCl$_2$ or CaCl$_2$ was added showed correlating shifts in the reversal potential. Here, we thus analyzed Ca$^{2+}$ and Mg$^{2+}$ selectivity among five HKT transporters, TaHKT2;1, AtHKT1;1, OsHKT2;1, OsHKT2;2 and OsHKT2;4. In addition to the confirmation of Ca$^{2+}$ and Mg$^{2+}$ permeabilities of OsHKT2;4 as reported (Lan et al., 2010), TaHKT2;1 was found to show a permeability for Mg$^{2+}$, and a possible effect of Ca$^{2+}$ on ionic currents (Fig. 2.6D-E). To gain insight into the significance of divalent cation transport activity by TaHKT2;1 and OsHKT2;4, competition experiments were performed in the presence of primary monovalent and divalent cation substrates. OsHKT2;4 and TaHKT2;1 both showed very small divalent cation permeabilities when 50 mM Ca$^{2+}$ or Mg$^{2+}$ were added to Na$^+$ and/or K$^+$ containing bath solutions (Fig. 2.8). These results suggest that these HKT transporters favor transport of K$^+$ and Na$^+$ over Ca$^{2+}$ and Mg$^{2+}$. Thus given the abundance of K$^+$ in plant cells, the physiological functions of OsHKT2;4 will depend strongly on the ionic conditions and the cell types in which OsHKT2;4 is expressed. Whether significant OsHKT2;4-mediated Ca$^{2+}$ or Mg$^{2+}$ transport activities occur in vivo in rice will need to be analyzed in planta, using rice knock out lines, similar to OsHKT2;1 analyses (Horie et al., 2007).

The OsHKT2;4 gene was reported to be diversely expressed in rice plants, including leaf sheaths and primary/lateral roots (Lan et al., 2010). Our findings suggest that OsHKT2;4 functions as a K$^+$-permeable transporter/channel with a smaller and competitively inhibited permeability to
Ca\(^{2+}\) and Mg\(^{2+}\). We have isolated two independent oshkt2;4 rice knock out lines from the Tos17 mutant population (Hirochika et al., 1996; Miyao et al., 2003; Horie et al., 2007), but no profound phenotype to various ionic conditions has been observed thus far (data not shown). OsHKT2;4 is highly identical to OsHKT2;3, including their 5' UTRs which also show conservation, and they could cooperatively function in vivo in rice plants. Further investigations of not only oshkt2;4 single mutants but oshkt2;3 single and oshkt2;3 oshkt2;4 double mutants could contribute to the understanding of the physiological roles of OsHKT2;4 in rice plants. Moreover, given the numerous studies analyzing the monovalent cation transporting properties of HKT transporters, and only two studies reporting analyses of divalent cation transport properties of HKT transporters (Lan et al., 2010 and the present study), further studies on the regulation and ion selectivities of both OsHKT2;3 and OsHKT2;4 will be important for assessing their biological potential for diverse physiological functions.

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2.6 References:


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