

# Channel-Like Characteristics of the Low-Affinity Barley Phosphate Transporter PHT1;6 When Expressed in *Xenopus* Oocytes<sup>1</sup>[W][OA]

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Remobilization of inorganic phosphate ( $P_i$ ) within a plant is critical for sustaining growth and seed production under external  $P_i$  fluctuation. The barley (*Hordeum vulgare*) transporter HvPHT1;6 has been implicated in  $P_i$  remobilization. In this report, we expressed HvPHT1;6 in *Xenopus laevis* oocytes, allowing detailed characterization of voltage-dependent fluxes and currents induced by HvPHT1;6. HvPHT1;6 increased efflux of  $P_i$  near oocyte resting membrane potentials, dependent on external  $P_i$  concentration. Time-dependent inward currents were observed when membrane potentials were more negative than  $-160$  mV, which was consistent with  $nH^+ : HPO_4^{2-}$  ( $n > 2$ ) cotransport, based on simultaneous radiotracer and oocyte voltage clamping, dependent upon  $P_i$  concentration gradient and pH. Time- and voltage-dependent inward currents through HvPHT1;6 were also observed for  $SO_4^{2-}$ , and to a lesser degree for  $NO_3^-$  and  $Cl^-$ , but not for malate. Inward and outward currents showed linear dependence on the concentration of external  $HPO_4^{2-}$ , similar to low-affinity  $P_i$  transport in plant studies. The electrophysiological properties of HvPHT1;6, which localizes to the plasma membrane when expressed in onion (*Allium cepa*) epidermal cells, are consistent with its suggested role in the remobilization of  $P_i$  in barley plants.

Phosphorus (P) is an essential macronutrient for plant growth and development, being required in the synthesis of nucleic acids, phospholipids, and ATP (Schachtman et al., 1998; Mudge et al., 2002; Lambers et al., 2006). The concentration of inorganic phosphate ( $P_i$ ) in the cytoplasm of plant cells is maintained at 5 to 17 mM (Schachtman et al., 1998; Mimura, 1999) while it rarely exceeds  $10 \mu M$  in the soil solution (Goldstein et al., 1988; Mimura, 1999). Agricultural crops heavily rely upon the application of P fertilizers for high yields (Morgan, 1997; Shenoy and Kalagudi, 2005; Lambers et al., 2006). P fertilizers are produced from nonrenewable deposits, and they are predicted to be half-depleted within 50 to 70 years (Oelkers and Valsami-Jones, 2008). Repeated application of P fertilizers can also result in significant environmental pollution (Sharpley et al., 1994; Molen et al., 1997).

The most promising way to reduce the dependence upon P fertilizers is to improve crop P use efficiency

(Graham and Welch, 1996; Shenoy and Kalagudi, 2005). This may be achieved by increasing  $P_i$  uptake or increasing the efficiency of  $P_i$  remobilization within the plant (Shenoy and Kalagudi, 2005). Remobilization of  $P_i$  is considered as an extremely important strategy for plant productivity in the environments where there is a large fluctuation in  $P_i$  supply (Papakosta, 1994; Rae et al., 2003).  $P_i$  remobilization is required for root growth under  $P_i$ -limiting environments (Marschner et al., 1996; Jeschke et al., 1997), and therefore affects root  $P_i$  uptake capacity (Shin et al., 2006), acting as a negative feedback control mechanism (Drew and Saker, 1984; Marschner et al., 1996; Peng and Li, 2005).  $P_i$  remobilization from older/shaded leaves also allows continued growth of younger and more photosynthetically active leaves (Jeschke et al., 1997), and it accounts for the majority, if not all of the P found in cereal grains (Horst et al., 1996; Masoni et al., 2007). Plant  $P_i$  transporters are involved in the movement of  $P_i$  within the cell and around the plant, and have been separated into families based on their predicted membrane localization: PHT1 (plasma membrane), PHT2 (plastid inner envelope), PHT3 (mitochondrial inner membrane), PHT4 (chloroplasts, heterotrophic plastids, and Golgi), and pPT (plastid inner envelope; Guo et al., 2008).

Plasma membrane  $P_i$  transporters (PHT1) are characterized as either high or low affinity and play a critical role in  $P_i$  movement between cells or tissues (Rae et al., 2003). Although much is known about high-affinity  $P_i$  transporters involved in  $P_i$  uptake by roots, little is currently known about the  $P_i$  transporters involved in remobilization.

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The  $P_i$  transporter *HvPHT1;6* is highly expressed in old leaves compared to young leaves, and its transcript is abundant in phloem tissue, with a lower level also observed in mesophyll cells (Rae et al., 2003). The expression of *HvPHT1;6* in both shoots and roots is up-regulated by P deficiency, and *HvPHT1;6* is the only *HvPHT1* family member found so far that is expressed in the shoots with high abundance (Rae et al., 2003; Huang et al., 2008). In addition, overexpression of *HvPHT1;6* in rice (*Oryza sativa*) suspension cells suggests it may have a high  $K_m$  or linear uptake kinetics (above wild-type  $P_i$  transport) to 1 mM external  $P_i$  (Supplemental Material S1). These data suggest that *HvPHT1;6* plays a major role in  $P_i$  remobilization.

Until recently, heterologous expression of plant  $P_i$  transporters in yeast (*Saccharomyces cerevisiae*) mutants lacking high-affinity  $P_i$  transporters (Pho84 and Pho89) have been the main means for functional characterization of both high- and low-affinity plant  $P_i$  transporters (Muchhal et al., 1996; Leggewie et al., 1997; Daram et al., 1998; Guo et al., 2008; Liu et al., 2008). In two cases, plant suspension cells have been used for functional characterization of plant  $P_i$  transporters (Mitsukawa et al., 1997; Rae et al., 2003); however, detailed electrophysiology has not been performed. It is difficult to perform electrophysiological measurements in yeast, as well as to isolate specific plant cell types for electrophysiological measurements. *Xenopus laevis* oocytes have been used for characterization of various  $P_i$  transporters including mammalian sodium (Na)- $P_i$  cotransporters and a plant  $P_i$  transporter (Bacconi et al., 2005; Ai et al., 2009). The rice phosphate transporter OsPT2 failed to complement yeast with a defect in high-affinity  $P_i$  transporters, but when expressed in *X. laevis* oocytes membrane depolarization could be observed in the presence of  $PO_4^{2-}$  (Ai et al., 2009). However, voltage dependence of proposed  $P_i$  transport, or potential interactions with other ions, was not investigated.

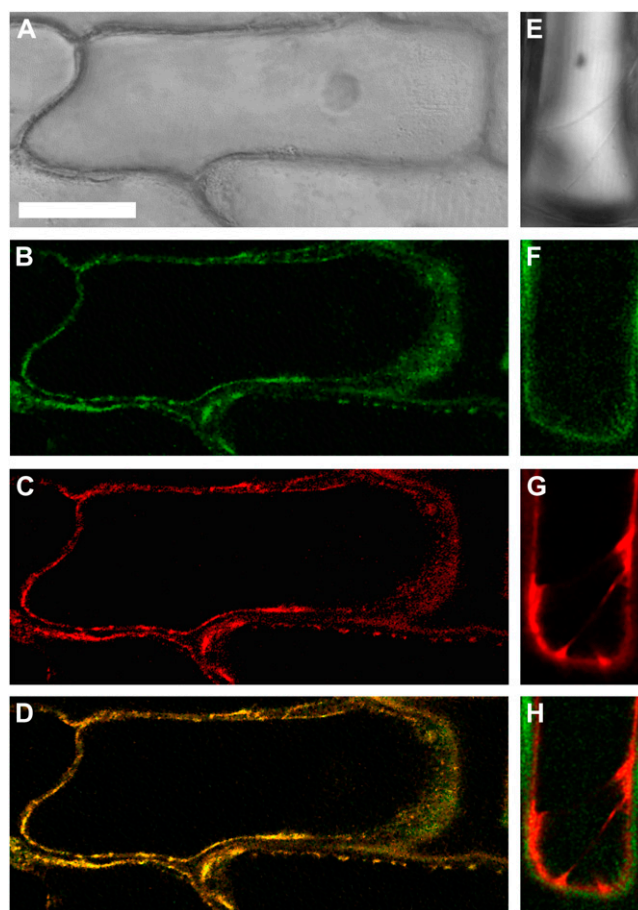
In this study, we confirm a plasma membrane localization of *HvPHT1;6*, and present a comprehensive analysis of *HvPHT1;6* transport activities using the *X. laevis* oocyte expression system. Our results demonstrate that *HvPHT1;6* is most likely to be a proton-coupled  $P_i$  transporter, though it has time-dependent activation at negative membrane potentials with linear concentration dependence similar to voltage-dependent ion channels; it also appears to transport  $SO_4^{2-}$  coupled to protons. As such, we discuss our results in the context of the proposed role of *HvPHT1;6* in  $P_i$  and sulfur (S) remobilization within the plant.

## RESULTS

### Plasma Membrane Localization of *HvPHT1;6*

*HvPHT1;6* mediates  $P_i$  transport into rice suspension cells (Rae et al., 2003), but its plasma membrane localization has not been established. Therefore, we

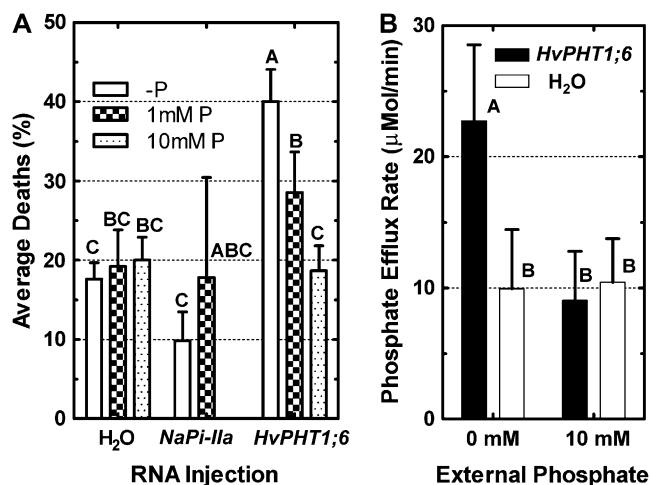
created a *HvPHT1;6::GFP* construct and transiently coexpressed with either the plasma membrane targeted marker, *AtPIP2A::mCherry*, or the vacuolar membrane marker,  *$\gamma$ TIP::mCherry* (Nelson et al., 2007), in onion (*Allium cepa*) epidermal cells. The green fluorescence of *HvPHT1;6::GFP* (Fig. 1B) colocalized with the red fluorescence of the plasma membrane marker pmrk (Fig. 1, C and D). In addition, the cellular location of the green fluorescence of *HvPHT1;6::GFP* (Fig. 1F) was separated from that of the red fluorescence of the vacuolar marker (Fig. 1, G and H), under hypertonic treatment. The red fluorescence of the vacuolar membrane marker was slightly intracellular compared to the green fluorescence of *HvPHT1;6::GFP* (Fig. 1H). These results indicate that *HvPHT1;6* is located on the plasma membrane.



**Figure 1.** Intracellular localization of *HvPHT1;6*. A, Bright-field image of onion epidermal cells. B, The intracellular localization of *HvPHT1;6::GFP* in onion epidermal cells. C, The intracellular localization of the plasma membrane marker, *AtPIP2A::mCherry*. D, Overlay of *HvPHT1;6::GFP* and *AtPIP2A::mCherry*; colocalization shown in yellow. E, Bright-field image of a second onion epidermal cell. F, The intracellular localization of *HvPHT1;6::GFP* in this cell. G, The intracellular localization of the vacuolar membrane marker,  *$\gamma$ TIP::mCherry* fusion. H, Overlay of the *HvPHT1;6::GFP* and  *$\gamma$ TIP::mCherry* fusion, showing differing localization of green and red fluorescence. Bar = 100  $\mu$ m.

### Effects of HvPHT1;6 on Mortality of *X. laevis* Oocytes

Injection of *HvPHT1;6* cRNA into *X. laevis* oocytes resulted in a 2-fold increase in oocyte deaths relative to water-injected control oocytes over the same time when incubated in modified Barth's solution (MBS; Fig. 2A). Addition of 1 mM or 10 mM  $P_i$  to the MBS solution significantly decreased mortality of *HvPHT1;6* cRNA-injected oocytes (Fig. 2A). No such relationship was observed in water-injected oocytes, nor for the positive control, oocytes expressing a human Na- $P_i$  transporter. This human Na- $P_i$  transporter has previously been shown to transport  $P_i$  into oocytes coupled with the downhill influx of  $Na^+$  (Bacconi et al., 2005; Virkki et al., 2005), and we are also able to show its electrogenic  $P_i$  transport activity (data not shown). *HvPHT1;6*-injected oocytes also had a higher efflux rate of  $P_i$  compared to controls when no  $P_i$  was added to the bath solution (Fig. 2B). This difference in efflux was abolished when 10 mM external  $P_i$  was applied (Fig. 2B), which was correlated with the reduced mortality of *HvPHT1;6*-injected oocytes incubated in MBS + 10 mM  $P_i$  (Fig. 2A). The application of  $P_i$  to the bath solution reduces free  $Ca^{2+}$  concentrations, which could have an impact on oocyte mortality. To discount this possibility we examined the effect of addition of 0.5 mM and 2 mM external calcium. There was no significant difference in oocyte survival or transport current ( $P > 0.7$  for each test,  $n = 10$ ). These results suggest that the increased death rate in *HvPHT1;6*-injected oocytes is due to the enhanced  $P_i$  efflux.



**Figure 2.** External phosphate reduces death of *HvPHT1;6* cRNA-injected oocytes. A, Percentage of oocyte deaths incubated in pH 7.5 MBS, containing three  $P_i$  ( $P$ ) concentrations ( $KH_2PO_4$ ), over 3 to 5 d at 18°C. Columns are means of three to eight replicates. B,  $H_3^{32}PO_4$  efflux rate. Oocytes were incubated for 24 h in MBS buffer with 10 mM 850 cpm  $nmol^{-1}$   $P_i$  before measuring the efflux rate in MBS over time. Data were corrected for radioactivity in wash solution. Standard errors ( $n = 4$ ) are shown as vertical bars. Different letters indicate a significance difference at  $P_{0.05}$  for confidence interval.

### Time- and Voltage-Dependent Inward Currents Induced by HvPHT1;6

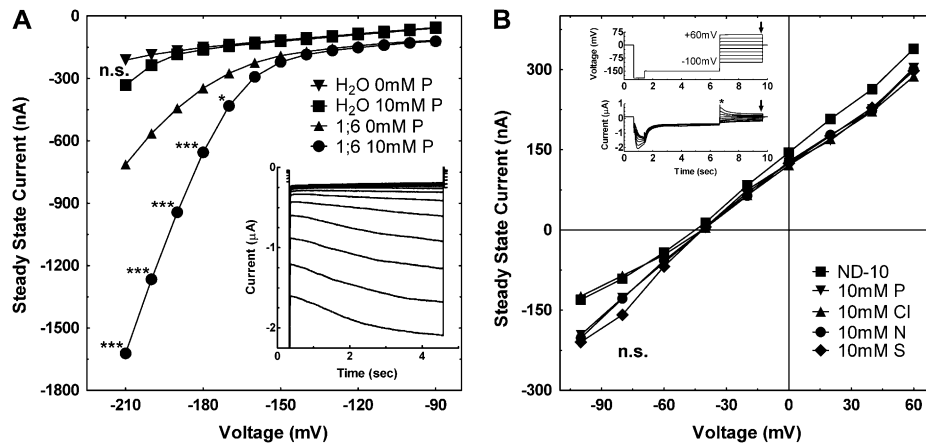
A bath solution containing 100 mM  $Cl^-$  (ND-100) was used for characterization of mammalian  $P_i$  transporters in *X. laevis* oocytes (Bacconi et al., 2005). Large inward currents were detected in *HvPHT1;6*-injected oocytes when ND-100 was used as a bath solution (data not shown). Water-injected control or cRNA-injected oocytes in solutions containing low amounts of  $Cl^-$  had a much lower inward current. Therefore, we used a 10 mM NaCl base solution (ND-10) generally (see "Materials and Methods") to which anions were balanced with *N*-methyl-D-glucamine (NMDG).

Under two-electrode voltage clamp (TEVC), negative-going voltage pulses caused the activation of a time- and voltage-dependent inward current that was increased by adding external  $P_i$  and was not observed in water-injected control oocytes (Fig. 3A). The example shown in Figure 3A is for 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-injected *HvPHT1;6*-expressing oocytes bathed in a modified ND-10 with  $Ba^{2+}$  replacing  $Ca^{2+}$  (Fig. 3A).  $Ba^{2+}$  and BAPTA were used to circumvent possible disturbance to the cytosolic  $Ca^{2+}$  concentration potentially caused by *HvPHT1;6* expression as such changes in cytosolic  $Ca^{2+}$  concentration could elicit native oocyte inward currents (Weber et al., 1995). *HvPHT1;6*-induced currents in these conditions were statistically the same as in non-BAPTA-injected oocytes ( $P > 0.7$ ), and those in calcium-bath solutions ( $P = 0.5$ ). Therefore, these treatments were not used for the majority of the following experiments. The activation of known endogenous oocyte channels was compared with that induced by *HvPHT1;6* cRNA injection (Supplemental Material S2). *HvPHT1;6* expression elicits currents different from native channels identified in the literature. Closer inspection of currents at less-hyperpolarized voltages with 10 mM  $P_i$ ,  $SO_4^{2-}$ , or  $NO_3^-$  in the bath solution revealed that steady-state inward currents began to be significantly activated at voltages more negative than about  $-90$  mV (when voltage was held constant for 4.5 s; Fig. 3).

When the voltage was taken to less-negative values after fully activating the inward current at  $-150$  mV, the current deactivated to a steady level with a half-time of  $0.33 \pm 0.05$  s (Fig. 3B). The difference in current between the initial and steady-state value during deactivation provides the time-dependent component of the *HvPHT1;6* cRNA-induced current at various voltages. We refer to this as the tail current. The majority of the following figures show results obtained from such tail analysis.

### Gradient-Dependent Currents and Fluxes

To further examine the effect of altering the gradient of  $P_i$  across the plasma membrane on the *HvPHT1;6*-induced tail currents, we injected water or  $P_i$  into *HvPHT1;6*-injected oocytes to a final calculated concentration of 10 mM and compared this with water-



**Figure 3.** *HvPHT1;6*-cRNA injection-induced inward current at hyperpolarized membrane potentials. A, Current/voltage curves of *HvPHT1;6*-cRNA, and water-injected oocytes. Oocytes were also BAPTA injected and bathed in ND-10 with Ba<sup>2+</sup> replacing Ca<sup>2+</sup> (pH 7.5). Values are the most negative current from a 4.2 s voltage hold (−90 to −210 mV in 10 mV increments). Data points are means of eight oocytes. Asterisks indicate a significant difference using Tukey's test ( $P_{0.05}$  or  $P_{0.001}$ ) between currents induced in 0 and 10 mM external NMDG-phosphate concentration. The inset shows overlaid current traces at each voltage of *HvPHT1;6* cRNA-injected oocytes bathed in 10 mM P<sub>i</sub>. B, Steady-state current/voltage curves extracted from tail current end point (arrows in the inset) comparing different external anions on *HvPHT1;6*-injected oocytes in ND-10, pH 7.5. Data points are means of at least five oocytes, no significant difference (n.s.) was observed ( $P_{0.05}$ ). Inset shows voltage protocol and example tail current trace overlay. Arrows indicate the tail end point, while currents marked at the asterisk time point are subtracted from the end point for tail current/voltage curves. P, Phosphate; Cl, chloride; N, nitrate; S, sulfate.

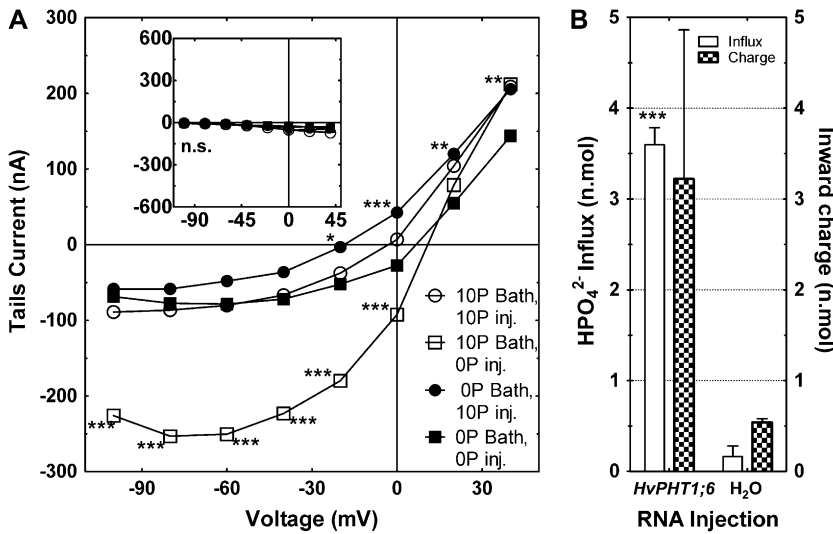
injected oocytes, bathed in either 0 or 10 mM P<sub>i</sub> added to ND-10. The largest inward tail currents at negative voltages were obtained when there was the largest gradient for inward movement of P<sub>i</sub> (0 P<sub>i</sub> injected, 10 mM P<sub>i</sub> in bath; Fig. 4A). Inward tail currents were smallest when this gradient was reversed (10 mM P<sub>i</sub> injected, 0 P<sub>i</sub> added to bath; Fig. 4). With 10 mM P<sub>i</sub> injected and 10 mM in the bath, the tail current/voltage curve reversed at 0 mV (Fig. 4A) under these conditions where the bath pH was 7.5. We measured <sup>32</sup>P uptake into *HvPHT1;6*-injected and control oocytes with voltage clamped at −130 mV for 7.5 min (Fig. 4B). The influx was increased 22-fold above that measured in control oocytes, and corresponded to a similar increase in the amount of charge that moved into the oocyte (Fig. 4B). Therefore, the currents associated with P<sub>i</sub> influx were accompanied by a flow of net-positive charge inwards to the oocyte. The same phenomenon was observed when <sup>35</sup>S was applied (Fig. 7B).

### Concentration Dependence and Selectivity

*HvPHT1;6*-injected oocytes showed a significant P<sub>i</sub>-induced increase in both inward and outward tail currents (Fig. 5). Corresponding with this was a small but significant shift in reversal potential toward positive voltages. The mean tail current as a function of added P<sub>i</sub> in the bath was linear in the range of external P<sub>i</sub> concentrations tested (Fig. 4B). Note that for a simple anion channel response it would be expected that the reversal potential would shift negative with increasing P<sub>i</sub> inward directed gradient (see also Fig. 4).

Selectivity of *HvPHT1;6*-induced currents to different anions was investigated by measuring steady-state currents at −150 mV (Fig. 6A), a physiologically relevant resting membrane potential for plant cells. The selectivity at −150 mV was HPO<sub>4</sub><sup>2−</sup> > SO<sub>4</sub><sup>2−</sup> > NO<sub>3</sub><sup>−</sup> > Cl<sup>−</sup>, where the external free anion concentration applied was kept constant at 9.9 mM (calculated using Visual MINTEQ, Royal Institute of Technology, Sweden). The organic anions, malate and citrate were also tested, but *HvPHT1;6*-injected oocytes showed no significant transport of these two organic anions over water-injected controls ( $P > 0.8$ ; data not shown). Bis-Tris propane (BTP) control solution induced less inward current than ND-10, which could be due to residual chloride transport through *HvPHT1;6* in ND-10. Also slight but significantly higher currents were seen in water-injected oocytes in the BTP solution (as opposed to the ND-10-based solutions), presumably due to a large difference in chloride concentration between the internal oocyte (24–62 mM; Weber, 1999) and the external bath (0.6 mM). When tail currents were measured at less-negative membrane potentials (Fig. 6B) SO<sub>4</sub><sup>2−</sup> and P<sub>i</sub> gave equal inward currents, and NO<sub>3</sub><sup>−</sup> and Cl<sup>−</sup> gave smaller and similar currents. However, NO<sub>3</sub><sup>−</sup> gave larger outward tail currents than any other anion (Fig. 6B). No significant anion-induced tail currents were observed in water-injected controls (inset in Fig. 6B).

A P<sub>i</sub> and SO<sub>4</sub><sup>2−</sup> competition experiment was performed to examine if competition occurred for transport sites of *HvPHT1;6* between these two anions (Fig. 7A); the inward and outward tail currents were com-



**Figure 4.** HvPHT1;6-induced phosphate transport is cation coupled. A, Tail current/voltage analysis of *HvPHT1;6* cRNA-injected oocytes in response to 0 or 10 mM NMDG-phosphate in the pH 7.5 ND-10 bath solution combined with 0 or 10 mM or internal oocyte NMDG-phosphate at least 2 h after injection. The inset shows water-injected oocyte controls. n.s., No significant difference. B, The average current integrals and subsequent radioactive  $H_3^{32}PO_4$  influx with a 450 s long hold at  $-130$  mV in a nonflowing pH 7.5 ND-10 bath solution. Data points in each experiment represent means of four to eight oocytes. Error bars indicate SEM, and asterisks denote a significant difference using the Scheffe's test ( $P_{0.05, 0.01, 0.001}$ ).

pletely additive between the two anions. Plotting the currents against the sum of the anions ( $HPO_4^{2-} + SO_4^{2-}$ ) displayed a linear relationship (inset in Fig. 7A) with mean  $R^2$  values ranging from 0.97 to 1.00 across all voltages.

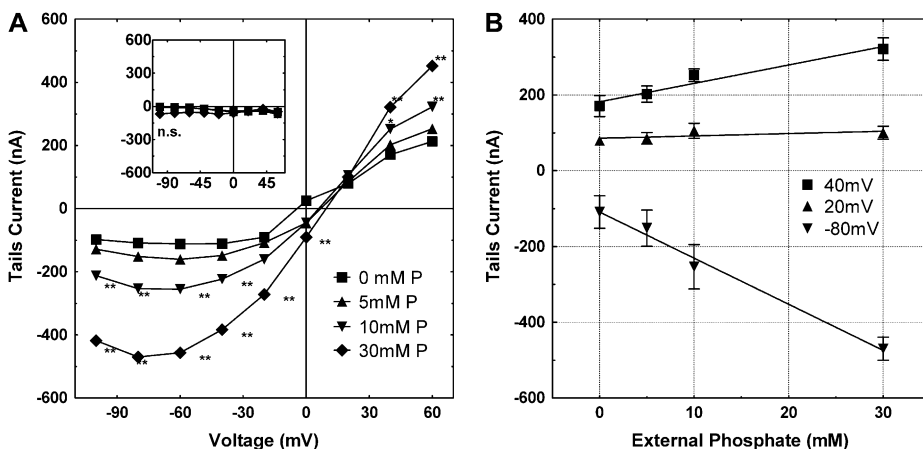
**External pH Sensitivity**

We examined the external pH ( $pH_o$ ) sensitivity of tail currents in  $P_i$  and  $SO_4^{2-}$  containing solutions, and no significant  $pH_o$  effect was detected in water-injected controls. Figure 8A shows that an increase in bath protons (pH decrease from pH 8 to 6) significantly increases the currents predicted to be  $SO_4^{2-}$ -influx. The change in current was linear over this pH range, increasing by a factor of  $2.02 \pm 0.18$  per pH unit. In contrast, *HvPHT1;6*-injected oocytes showed the largest  $P_i$ -induced inward currents at pH 7.5 (Fig. 8B). The general trend for  $P_i$  transport therefore opposes what was seen for  $SO_4^{2-}$ . However, the  $HPO_4^{2-}$  ion is predicted to decrease in free concentration as pH decreases (calculated using Visual MINTEQ). If we

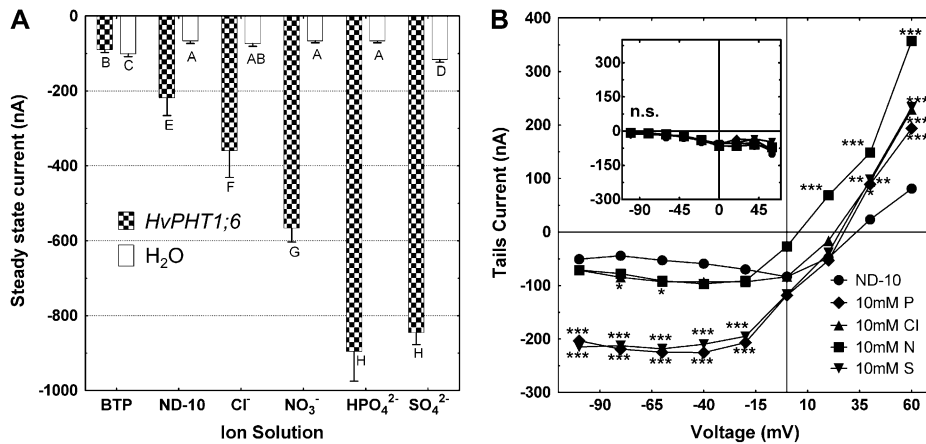
take account of the stimulating effect of lower pH on  $SO_4^{2-}$  currents, and make the assumption that the selectivity between  $HPO_4^{2-}$  and  $SO_4^{2-}$  does not change with pH, we can calculate predicted inward currents for  $HPO_4^{2-}$  as a function of pH (Fig. 8B). Within the confidence limits of the measured currents, the predicted currents fit reasonably well with  $HPO_4^{2-}$  being the transported form of  $P_i$ .

**DISCUSSION**

Despite the importance of low-affinity  $P_i$  transporters in  $P_i$  remobilization in higher plants, the functional characterization of low-affinity  $P_i$  transporters from plants has been limited to either the complementation of yeast mutants defective in high-affinity  $P_i$  permeases (Daram et al., 1998; Miller and Zhou, 2000; Guo et al., 2008; Liu et al., 2008) or ectopic expression of low-affinity  $P_i$  transporters in rice (Rae et al., 2003) or tobacco (*Nicotiana tabacum*; Mitsukawa et al., 1997) suspension cells. One study in *X. laevis*



**Figure 5.** *HvPHT1;6* transports phosphate with low affinity. A, Current/voltage tail plot of *HvPHT1;6*-injected oocytes bathed in ND-10 with different phosphate concentrations at pH 7.5. The inset shows water-injected oocyte responses. n.s., No significant difference. Asterisks indicate a significant difference at  $P_{0.05}$  and  $P_{0.01}$  using Tukey's test. B, *HvPHT1;6*-injected oocyte tail analysis at selected voltages. Error bars indicate SEM of four oocytes.



**Figure 6.** Anion selectivity of HvPHT1;6. A, Steady-state current response to six different bath solutions: BTP control, ND-10 control, ND-10 plus 9.9 mM transported oxyanion (actual applications were 10 mM), NMDG-chloride ( $\text{Cl}^-$ ), 10 mM NMDG-nitrate ( $\text{NO}_3^-$ ), 14.88 mM NMDG-phosphate ( $\text{HPO}_4^{2-}$ ), or 11 mM NMDG-sulfate ( $\text{SO}_4^{2-}$ ). Steady-state currents were recorded after 5 s at  $-150$  mV. Columns represent means of five oocytes, and error bars indicate SEM. Different letters indicate significant differences at  $P_{0.05}$  via confidence interval. B, Tail current/voltage responses of HvPHT1;6-injected and water-injected oocytes run in a ND-10 bath (control), or containing either NMDG-phosphate (P), NMDG-chloride (Cl), NMDG-nitrate (N), or NMDG-sulfate (S). pH was buffered with Tris-base to pH 7.5. The inset shows the response of water-injected controls. n.s., No significant difference. Asterisks indicate a significant difference at  $P_{0.05, 0.01, 0.001}$  (Tukey's test) between the respective anion treatment and the control.

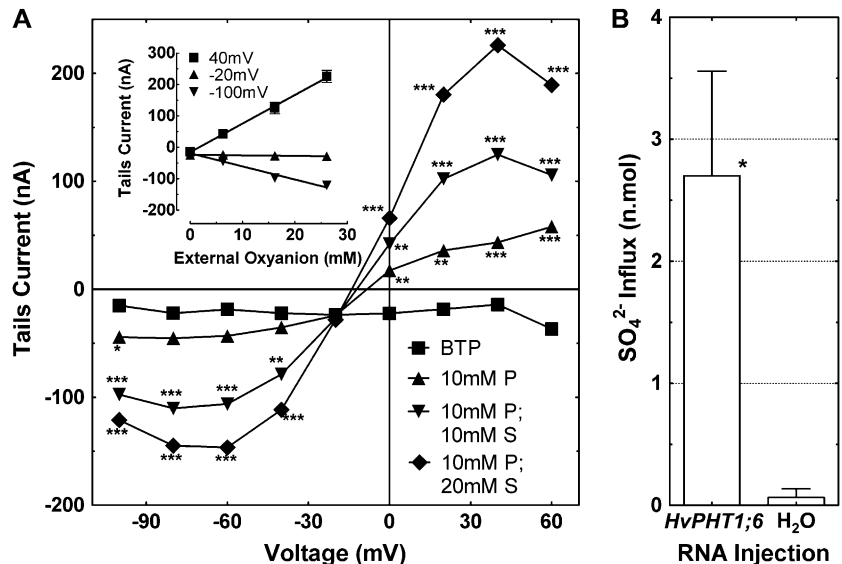
oocytes of a plant  $\text{P}_i$  transporter (OsPHT1;2) shows only limited information on membrane depolarizations when 1 or 10 mM  $\text{NaH}_2\text{PO}_4$  was applied, suggesting that OsPHT1;2 is a low-affinity  $\text{P}_i$  transporter (Ai et al., 2009).

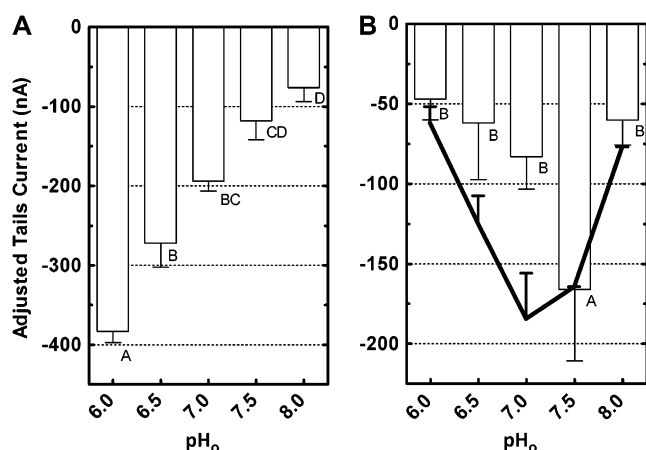
We first show that HvPHT1;6 is expressed in the plasma membrane of plant cells (Fig. 1), secondly that HvPHT1;6 expression is not just activating a known native transporter in the plasma membrane of *X. laevis* oocytes (Supplemental Material S2). This information gives relevance to HvPHT1;6 being involved in  $\text{P}_i$

movement between plant cells/tissues and that characterization in *X. laevis* oocytes is appropriate.

Expression of the low-affinity HvPHT1;6 transporter in *X. laevis* oocytes with no supplementation of  $\text{P}_i$  in the external medium caused increased deaths of the oocytes (Fig. 2A). Based on  $^{32}\text{P}_i$  efflux measurements (Fig. 2B) we interpret the increased deaths of the oocytes as a result of increased  $\text{P}_i$  efflux caused by expression of HvPHT1;6. The resting membrane potential for HvPHT1;6-injected oocytes in ND-100 solution was  $-26.8 \pm 2.6$  mV that is not different from the

**Figure 7.** Phosphate and sulfate competition. A, Tail current/voltage plot of HvPHT1;6-injected oocytes between the BTP control solution, and the BTP containing 10 mM  $\text{H}_3\text{PO}_4$  (P) with or without 10 or 20 mM  $\text{H}_2\text{SO}_4$  (S). pH was buffered with Tris-base to pH 7.5. The inset shows a linear regression of selected currents against calculated free  $\text{HPO}_4^{2-}$  or  $\text{SO}_4^{2-}$  in the bath solution ( $r^2 > 0.96$ , Visual MINTEQ). Data points indicate means of seven individual oocyte measurements. B, Radioactive  $\text{Na}_2^{35}\text{SO}_4$  influx with a 450 s long hold at  $-130$  mV in a stagnant pH 7.5 ND-10 bath solution. Columns are means of five oocytes. Error bars indicate SEM, and asterisks denote a significant difference using Tukey's test ( $P_{0.05, 0.01, 0.001}$ ).





**Figure 8.** Proton-coupled  $P_i$  transport activity of HvPHT1;6. HvPHT1;6-induced outward and inward currents in response to sulfate (A) or phosphate (B). Columns indicate mean currents of eight oocytes for phosphate transport via HvPHT1;6 only, using a dual subtraction process: (1) subtracting water-injected oocyte currents from HvPHT1;6 injected, then (2) subtracting ND-10 control solution currents from ND-10 plus 10 mM NMDG-phosphate or sulfate currents at each pH<sub>o</sub>. Letters indicate significant difference ( $P_{0.05}$ , Tukey's test), and error bars represent SEM. Predicted  $HPO_4^{2-}$  currents (solid line in B) accounting for changes in  $[HPO_4^{2-}]$  with pH<sub>o</sub> were calculated from the  $SO_4^{2-}$  currents assuming that the transport selectivity between  $HPO_4^{2-}$  and  $SO_4^{2-}$  does not change with pH<sub>o</sub>. Predictions are only given for influx because accurate estimation of internal oocyte activity of  $HPO_4^{2-}$  and  $SO_4^{2-}$  could not be made.

resting membrane potential in MBS of  $-29.2 \pm 9.5$  mV, this could be a reason why  $P_i$  efflux occurs in the absence of external  $P_i$ . Data in Figure 4A show that under conditions of high internal  $P_i$ , and with zero in the bath, the reversal potential of HvPHT1;6-injected oocytes, as indicated by tail currents, is close to these values. When  $P_i$  is added to the bath, the reversal potential shifts positive, which we predict would reduce  $P_i$  efflux from the oocyte (see below for more discussion in "Stoichiometry of  $H^+$ - $P_i$  Cotransport and Speciation"). In plant cells, efflux of  $P_i$  through HvPHT1;6 under membrane depolarization could be related to efflux of  $P_i$  from leaf mesophyll cells when they start senescing, and is possibly why enhanced expression is seen in older leaf mesophyll cells (Rae et al., 2003). However, this would require neutral to alkaline pHs in the external medium as used in our oocyte experiments.

### Voltage Dependence

Activation of inward current in HvPHT1;6-injected oocytes occurred when voltages were more negative than  $-60$  mV, and became highly significant between  $-130$  and  $-160$  mV (Fig. 3). This is a rather novel finding, but is not totally unexpected, because while the resting potential of an oocyte is usually around  $-30$  to  $-40$  mV, the equivalent value for a plant cell can be less than  $-200$  mV (Dreyer et al., 1999), and

phloem cells are more hyperpolarized than their surrounding cells (Hafke et al., 2005). The hyperpolarization in phloem cells should be able to activate HvPHT1;6 to facilitate  $P_i$  loading in phloem-associated cells for future remobilization. In many respects the slow kinetics of voltage activation of HvPHT1;6 observed in oocytes is similar to some plant ion channels (Marten et al., 1999). The linear concentration transport kinetics would further support that HvPHT1;6 possesses characteristics of an ion channel. However, the evidence that inward current corresponds to anion influx instead of efflux suggests  $P_i$  through HvPHT1;6 is cotransported with a net positive charge, therefore HvPHT1;6 is a class of transporter with features in common between a channel and a transporter.

### Low-Affinity Transport

HvPHT1;6 showed a linear transport activity for  $P_i$ -stimulated inward current over a concentration range of 5 to 30 mM. This concentration range encompasses what the transporter may be exposed to in planta (Schachtman et al., 1998). An apparent linear  $P_i$  uptake in a concentration range of 0 to 1 mM can also be interpreted from the data of Rae et al. (2003; Supplemental Material S1) who observed higher  $P_i$  influxes in rice suspension cells overexpressing HvPHT1;6 but with similar  $K_m$  to control cells. The linear  $P_i$  uptake kinetics in the oocytes matches with the function of the low-affinity  $P_i$  transport in barley (*Hordeum vulgare*) leaves (Mimura, 1999) that is required for  $P_i$  remobilization.

### Stoichiometry of $H^+$ - $P_i$ Cotransport and Speciation

It is widely accepted that  $P_i$  uptake in higher plants is coupled to net cation cotransport as  $P_i$  absorption depolarizes the plasma membrane of root cortex cells (Mimura, 1999). However, the accurate stoichiometry of  $X^+$ - $P_i$  cotransport has not been obtained as yet. By changing the  $P_i$  gradients we have demonstrated that when the  $P_i$  concentration gradient across the plasma membrane favors higher  $P_i$  influx, it produces a larger inward current (Fig. 4). The reversal potentials also shift positive with increasing gradient for inward flux of  $P_i$ . We also showed that  $^{32}P$  influx at negative voltages corresponded to the total charge taken up (Fig. 5). Therefore,  $P_i$  influx must be coupled with cation influx with a net positive charge entering the cell.

Mammalian  $P_i$  transporters are coupled with  $Na^+$  (Bacconi et al., 2005). However, HvPHT1;6 did not use  $Na^+$  or  $K^+$  as a driver cation because the currents induced by  $P_i$  and  $SO_4^{2-}$  were observed, to the same degree ( $P > 0.43$ ,  $n = 7$ ), in the absence of  $Na^+$  and  $K^+$  in the bath solution (Fig. 7A). On the other hand, pH was found to have a significant effect on the transport activity of HvPHT1;6-expressing oocytes, with the inward tail currents induced by  $SO_4^{2-}$  increasing with a decrease in pH. Therefore,  $H^+$  are likely to be the coupling cation to anion movement through

HvPHT1;6. Over the range of pH 6.0 to 8.0, the free concentration of  $\text{SO}_4^{2-}$  changed less than 0.1 mM (<1% of the total  $\text{SO}_4^{2-}$  concentration). In contrast,  $\text{P}_i$  transport increases from pH 6.0 to 7.5 and is reduced at pH 8.0 (Fig. 8B). Because the concentrations of different ionic forms of  $\text{P}_i$  in solution change with pH, the pH experiment provides evidence that the likely  $\text{P}_i$  form transported is  $\text{HPO}_4^{2-}$ . Using the change in inward current with  $\text{SO}_4^{2-}$  as a function of pH to represent the effect of pH on  $\text{HPO}_4^{2-}$  transport, we can predict how  $\text{pH}_o$  should affect inward current, taking into account the change in  $\text{HPO}_4^{2-}$  with a constant total  $\text{P}_i$  concentration of 10 mM. This assumes that  $\text{HPO}_4^{2-}$  and  $\text{SO}_4^{2-}$  transport behaves the same with pH and that both ions give a linear inward current response with concentration (Fig. 7A). The predicted values show a decrease in inward current with lowered pH as is observed in the experiment. The discrepancy at pH 7 and 6.5 are close to the 95% confidence limits of the measured values and the predicted values from the regression of  $\text{SO}_4^{2-}$  current as a function of pH. If  $\text{H}_2\text{PO}_4^-$  was the transported ion, the opposite effect would be observed, i.e. a concave-down increase in inward current with decreased pH. Previous work suggested  $\text{H}_2\text{PO}_4^-$  to be the ion transported by HvPHT1;6 (Rae et al., 2003), this however seems very unlikely as discussed above. The  $\text{HPO}_4^{2-}$  ion, on the other hand, shows a concentration change that aligns with the ionic current responses. Interestingly,  $\text{MgHPO}_4$  shows a similar concentration change as  $\text{HPO}_4^{2-}$  with pH. However,  $\text{MgHPO}_4 + \text{H}^+$  is unlikely to be transported through HvPHT1;6 as BAPTA injection reduces active Mg in the oocyte but efflux currents were the same as with no BAPTA injection, and significant influx of  $\text{P}_i$  (to the same degree,  $P > 0.43$ ,  $n = 7$ ) was observed in the complete absence of  $\text{Mg}^{2+}$  (Fig. 7A).

Inferences can be made on the transport stoichiometry for  $\text{H}^+$ -coupled  $\text{HPO}_4^{2-}$ . A comparison of chemical flux with total ion current from experiments used for Figure 4B gives a ratio of charge to  $\text{P}_i$  uptake of 1:1, i.e.  $3\text{H}^+ : 1\text{HPO}_4^{2-}$  (calculated on an individual oocyte basis). Radioactive  $\text{P}_i$  uptake in water-injected oocytes was not significantly different from zero, suggesting an absence of native  $\text{P}_i$  transporter activities in oocytes under our experimental conditions.

### Broad Selectivity of HvPHT1;6

HvPHT1;6 showed little selectivity between  $\text{HPO}_4^{2-}$  and  $\text{SO}_4^{2-}$  (Fig. 7A, and radioactive uptake calculations, Figs. 4B and 7B), with a lower transport activity for  $\text{NO}_3^-$  and  $\text{Cl}^-$  (Fig. 6A). There was no malate or citrate transport (data not shown). Limited information is available for comparison of anion selectivity in oocytes expressing plant transporters. It has been shown that GmN70, an anion transporter on the symbiosome membrane, favors  $\text{NO}_3^-$  transport (Vincill et al., 2005), and TaALMT1 favors malate transport but can be selective for  $\text{Cl}^-$  under certain conditions (Pineros et al., 2008). The  $\text{HPO}_4^{2-}$  anion has a dehy-

drated minimum ionic width of 2.518 Å, compared with  $\text{SO}_4^{2-}$  at 2.535 Å (100.6% of  $\text{HPO}_4^{2-}$ ),  $\text{NO}_3^-$  at a width of 2.148 Å (84.7% of  $\text{HPO}_4^{2-}$ ), and  $\text{Cl}^-$  with a width of 1.04 Å (41% of  $\text{HPO}_4^{2-}$ ; ACD/ChemSketch version 11.0, Advanced Chemistry Development, Inc.).  $\text{SO}_4^{2-}$  is very similar in size and charge density to  $\text{HPO}_4^{2-}$  (complete hydration of these ions makes  $\text{HPO}_4^{2-}$  a slightly larger molecule than  $\text{SO}_4^{2-}$ ) and has the same charge and oxyanion characteristics, which seem to be required for selective transport by HvPHT1;6.  $\text{NO}_3^-$  is also an oxyanion, but is smaller in size, and has only a single negative charge, whereas  $\text{Cl}^-$  is much smaller and does not have the oxygen. Therefore, the reduced transport of  $\text{NO}_3^-$  and  $\text{Cl}^-$  may be expected on this basis. Surprisingly, the electrophysiological experiments did not demonstrate any competition between  $\text{HPO}_4^{2-}$  and  $\text{SO}_4^{2-}$  when they were added together and we have shown  $^{35}\text{SO}_4$  uptake by HvPHT1;6-expressing oocytes without  $\text{P}_i$  present in the bath. However it does remain a possibility that external  $\text{SO}_4^{2-}$  may stimulate  $\text{HPO}_4^{2-}$  transport.

### Potential Roles of HvPHT1;6 in $\text{P}_i$ Remobilization

$\text{P}_i$  is tightly regulated at about 10 mM within plant cell cytoplasm (Schachtman et al., 1998). Nitrate concentration is more variable, between 3.4 to 37 mM (Siddiqi and Glass, 2002). Sulfate in the cytoplasm is at a concentration of approximately 2.5 mM (Cameron et al., 1984). The majority of these anions are removed from senescing leaves. In *Arabidopsis thaliana* 88% of nitrogen, 80% of P, and 68% of S are removed from senescing leaves (Himmelblau and Amasino, 2001). HvPHT1;6 shows the highest expression in the phloem cells of older leaves (Rae et al., 2003). Because HvPHT1;6 shows transport activity of  $\text{HPO}_4^{2-}$ ,  $\text{SO}_4^{2-}$ , and  $\text{NO}_3^-$  in oocytes (Fig. 6), it is possibly involved in the remobilization of these ions around the barley plant. Studies have shown rice phloem sap to consist of 8.1 mM  $\text{PO}_4^{3-}$ , 1.9 mM  $\text{NO}_3^-$ , and 1.8 mM  $\text{SO}_4^{2-}$  (Hayashi and Chino, 1985); and wheat (*Triticum aestivum*) phloem sap 8.2 mM  $\text{PO}_4^{3-}$ , 8.1 mM  $\text{NO}_3^-$ , and 1.0 mM  $\text{SO}_4^{2-}$  (Hayashi and Chino, 1986). Therefore, cereals have relatively high levels of these anions remobilized via the phloem. The relatively lower concentration of  $\text{SO}_4^{2-}$  remobilized could be due to its lower concentration in plant cells (Cameron et al., 1984) and/or its reduced level of removal from senescing leaves (Himmelblau and Amasino, 2001), rather than the ability of it to be loaded into the phloem. Remobilization can remove P, S, and nitrogen from older leaves into tissues where it is most needed, in wheat an average 52% to 100% of grain P (Papakosta, 1994) and 72% of grain nitrogen (Gooding et al., 2005) are sourced via remobilization from older plant tissues. It is not entirely clear what significance a low-affinity  $\text{P}_i$  transporter has in the remobilization of sulfate and nitrate in planta. Further experimentation is warranted; an interesting experiment would be analysis of HvPHT1;6 knockout mu-



tants for differences in remobilization of phosphate, sulfate, and nitrate from senescing leaves.

In summary, nutrient reserves deposited in vegetative plant parts before anthesis buffer grain yield against conditions adverse to assimilation during the grain-filling period. In wheat, remobilization accounts for the majority of grain P content and increases P use efficiency. Phloem tissues are responsible for trafficking nutrient remobilization; and because phosphate, nitrate, and sulfate cannot reach the phloem via the symplast, a transporter must exist in the membranes of barley phloem cells to transport these nutrients into the phloem. We show that HvPHT1;6 is targeted to the plasma membrane of plant cells and has the capacity to transport  $P_i$  coupled with protons in a highly voltage-dependent manner. HvPHT1;6 is a low-affinity  $P_i$  transporter with potential to transport other oxyanions. HvPHT1;6 could fulfil a role in  $P_i$  remobilization because it is highly expressed in phloem tissues. Our results demonstrate that *Xenopus* oocytes can be used for detailed characterization of plant  $P_i$  transporters, and this will facilitate structure-function studies of plant  $P_i$  transporters.

## MATERIALS AND METHODS

### Cloning of HvPHT1;6

Genomic DNA from barley (*Horedum vulgare* 'Clipper') was used to clone HvPHT1;6 using PCR with a pair of primers (ATGGCGCGGAG and TCACACGGGCACCG). PCR products were ligated into the pCR8-GW-TOPO vector (Invitrogen). The resulting HvPHT1;6 plasmid was sequenced for confirmation, and then HvPHT1;6 was transferred into a gateway-enabled pGEM-HE-DEST vector (Shelden et al., 2009) using the LR reaction protocol (Invitrogen) for in vitro RNA synthesis. The human *NaPi-IIa* positive control in the KSM expression vector (from Leila Virkki) was digested with *AccI* and *HindIII*, and then cloned into pGEM-HE.

### Subcellular Localization of HvPHT1;6::GFP

The coding sequence of HvPHT1;6 without the stop codon was amplified from pGEMHvPHT1;6:HE using the primer pair ATGGCGCGGAG and CACGGGCACCGTC. The PCR fragment was ligated into the pCR8-GW-TOPO vector, and transferred into pMDC83 containing the *mGFP* gene (Curtis and Grossniklaus, 2003). The resulting plasmid places HvPHT1;6 in frame, upstream of *mGFP6*.

Plasmid DNA: CD3-1007 (*AtPIP2A::mCherry* fusion), CD3-975 (*γTIP::mCherry* fusion), and pMDC83HvPHT1;6 (5 μg each and 10 μL in total volume) was mixed with 50 μL of 0.6 μm gold particles (Bio-Rad), and bombarded into onion (*Allium cepa*) epidermal cells (900 psi pressure rupture discs) using the Biolistic PDS-1000/He particle delivery system (Bio-Rad). Bombarded onion cells were kept in the dark at room temperature for 48 to 72 h and then examined by the confocal laser-scanning microscopy (Leica TCS SP5 spectral scanning confocal microscope). Onion epidermal cells were grown in Murashige and Skoog medium supplemented with 60 g L<sup>-1</sup> Suc. The onion epidermal cells were immersed in Murashige and Skoog medium supplemented with 100 g L<sup>-1</sup> Suc before confocal image analysis. GFP fluorescence was excited using the 488-nm argon laser, and mCherry fluorescence was excited using the 561-nm DPSS 561 laser.

### RNA Synthesis

In vitro RNA syntheses were performed on two separate occasions using the T7 RNA polymerase kit (Ambion) for HvPHT1;6 and *NaPi-IIa*. Synthesis was done at 37°C for 2 h, and the products were cleaned using phenol and chloroform according to manufacturer's instructions. The quality and size of synthesized RNA were checked on RNase-free agarose gels.

### Oocyte Extraction and Preparation

*Xenopus laevis* frogs (NASCO Biology) were anesthetized in 1 L ice-cold 1.5% (w/v) 3-aminobenzoic acid ethyl ester methanesulfonate salt for 20 min. Oocytes were removed unilaterally from the abdominal cavity, and the lobes were placed in calcium-free Frog Ringer's buffer (in mM: 96 NaCl, 2 KCl, 5 MgCl<sub>2</sub>, 5 HEPES, pH 7.6). The lobes were cut into small pieces and placed in 50 mL of calcium-free Frog Ringer's buffer containing 100 mg collagenase and 50 mg trypsin inhibitor for 85 to 90 min with rotation on a rotary mixer before being washed three times with hypotonic buffer (in mM: 100 KH<sub>2</sub>PO<sub>4</sub> pH 6.5 and 0.1% [w/v] bovine serum albumin). The oocytes were incubated in hypotonic buffer on a rotary shaker for 10 min at room temperature. Oocytes were then washed three times in calcium-free Frog Ringer's, followed by two washes in calcium Ringer's, a 10 min incubation on a rotary shaker, then two washes in calcium Ringer's. The oocytes were maintained at 18°C in MBS solution (in mM): 96 NaCl, 2 KCl, 5 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 5 HEPES, 10 KH<sub>2</sub>PO<sub>4</sub>, adjusted to pH 7.6 with KOH; 2.5 mL horse serum was added in 50 mL solution (catalog no. H1270), 50 mg mL<sup>-1</sup> tetracyclin (5 mg mL<sup>-1</sup> stock, used 0.5/50 mL), and 0.5 mL per 50 mL of penicillin streptomycin (catalog no. P4333). Healthy stage IV and V oocytes from 10 different oocyte batches were selected for injection with 25 ng RNA (i.e. 50 nL of 500 μg mL<sup>-1</sup> RNA). The injection into oocyte animal hemisphere was performed at room temperature using a Nanoject II injector (Drummon Scientific Company) and injected oocytes were incubated at 18°C in MBS (replacing daily) for 20 to 96 h prior to ion flux and electrophysiological measurements.

### Injection of Phosphate and Nitrate

All chemicals were sourced from Sigma-Aldrich unless stated otherwise. Healthy HvPHT1;6-expressing and water control oocytes (1 d after injection) were selected for injection with 50 nL water (control) or 114 mM NMDG-phosphate (or -nitrate) or 11.3 mM BAPTA, to a final concentration of phosphate (10 ± 0.19 mM) or BAPTA (1 ± 0.02 mM) in the oocyte. These measurements were based on an average (*n* = 100), and <sup>3</sup>H<sub>2</sub>O available internal volume of 570 ± 11 nL (Stegen et al., 2000). After phosphate and nitrate injection, oocytes were incubated at 18°C in MBS for 2 h before electrophysiological measurements.

### Solutions

Individual oocytes were selected for voltage clamp experiments. ND-10 bath solution (in mM: 10 NaCl, 80 mannitol, 2 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and pH 7.5 adjusted with Tris-base) was continuously running at 1.94 mL min<sup>-1</sup>. This bath solution allowed the addition of up to 30 mM NMDG-phosphate. Solutions adjusted to pH 6 and 6.5 had 10 mM MES instead of HEPES. ND-100 solution was same as ND-10 except that it contained 100 mM NaCl and no mannitol. The BTP solution consisted of 0.3 mM CaCl<sub>2</sub> (control), and when varying levels of phosphoric or sulfuric acid were added to the solution, pH was adjusted to 7.5 with BTP. All solutions were adjusted with mannitol to a final osmolarity of 205 mOsmol kg<sup>-1</sup>.

### Electrophysiology

TEVC experiments were performed with a GeneClamp500 amplifier under control of the Clampex8 program (Axon Instruments). Individual experiments were performed on one to six different batches of oocytes and showed exactly the same trend in each occasion. All figures presented in this article were data from oocytes 2 d after injection. Impaling electrodes were filled with 0.22 μm filtered 3 M KCl (0.5–1.0 M current-injecting electrode, and 1.0–2.0 M for voltage-sensing electrode). Oocytes were deemed acceptable if the stable resting membrane potentials were negative of -25 mV in ND-10. The voltage clamp protocol for current tail analysis was 0 mV for 0.5 s, -190 mV for 1 s, -150 mV for 5 s, then a differential voltage ranging 60 mV to -100 mV for 3 s in a -20 mV increment. This protocol was designed so that current activation in HvPHT1;6-cRNA-injected oocytes came to a similar saturated level before depolarizing steps.

### Phosphate Fluxes

Phosphate influx while simultaneously performing TEVC was measured in ND-10 containing 10 mM NMDG-phosphate. The load solution had H<sub>3</sub><sup>32</sup>PO<sub>4</sub>

(catalog no. NEX053001MC, Perkin Elmer) added to an average experimental specific activity of 463 cpm nmol<sup>-1</sup>. A chamber was constructed by indenting the base of a 35 mm diameter petri dish with a soldering iron to hold an individual oocyte. After the oocyte was impaled and TEVC initiated in nonradioactive solution, the solution was immediately replaced with the radioactive solution of an equal phosphate concentration for a 7.5 min voltage clamp at -130 mV. The radioactive solution was immediately replaced by nonradioactive ice-cold solution with 5 × 3 mL washes after the voltage clamp. Scintillation counting was conducted (S6500, multifunction scintillation counter, Beckman and Coulter) on 30 μL of radioactive solution, 30 μL final wash solution with the disintegrated oocyte, and 30 μL final wash solution with 4 mL IRGA-Safe Plus scintillation fluid (Perkin Elmer).

For phosphate efflux measurements, oocytes were incubated in radioactive solution (specific activity of 850 dpm nmol<sup>-1</sup>) for 24 h at 19°C, and then they were washed for 3 s in 100 μL cold solution. After that, radioactive efflux from oocytes was measured in 100 μL solution at time intervals of 3 s, 5, 10, 20, 40, 80, and 180 min. The remaining radioactivity in oocytes was also measured. Radioactive efflux from water-injected or *HvPHT1;6* cRNA-injected oocytes was also measured in the presence of 10 mM and 0 mM external NMDG-phosphate. A total of 100 μL of each sample was added to 4 mL scintillation fluid for radioactivity counting.

## Sulfate Influxes

Sulfate influx was performed exactly the same as phosphate influx, with the exceptions of using 5 mM NMDG-sulfate and a load solution containing Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (catalog no. NEX041H001MC, Perkin Elmer) added to an average experimental specific activity of 3,178 cpm nmol<sup>-1</sup>.

The *HvPHT1;6* nucleotide sequence was deposited in the EMBL database (accession no. FM866444).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Material S1.** Alternative interpretation of P<sub>i</sub> uptake when *HvPHT1;6* is expressed in rice suspension cells.

**Supplemental Material S2.** Comparison of *HvPHT1;6*-induced currents with native oocyte channels.

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