A Single-Pore Residue Renders the *Arabidopsis* Root Anion Channel SLAH2 Highly Nitrate Selective

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In contrast to animal cells, plants use nitrate as a major source of nitrogen. Following the uptake of nitrate, this major macronutrient is fed into the vasculature for long-distance transport. The *Arabidopsis thaliana* shoot expresses the anion channel SLOW ANION CHANNEL1 (SLAC1) and its homolog SLAC1 HOMOLOGOUS3 (SLAH3), which prefer nitrate as substrate but cannot exclude chloride ions. By contrast, we identified SLAH2 as a nitrate-specific channel that is impermeable for chloride. To understand the molecular basis for nitrate selection in the SLAH2 channel, SLAC1 and SLAH2 were modeled to the structure of Hi-TehA, a distantly related bacterial member. Structure-guided site-directed mutations converted SLAC1 into a SLAH2-like nitrate-selective anion channel and vice versa. Our findings indicate that two pore-occluding phenylalanines constrict the pore. The selectivity filter of SLAC/SLAH anion channels is determined by the polarity of pore-lining residues located on alpha helix 3. Changing the polar character of a single amino acid side chain (Ser-228) to a nonpolar residue turned the nitrate-selective SLAH2 into a chloride/nitrate-permeable anion channel. Thus, the molecular basis of the anion specificity of SLAC/SLAH anion channels seems to be determined by the presence and constellation of polar side chains that act in concert with the two pore-occluding phenylalanines.

**INTRODUCTION**

In the model plant *Arabidopsis thaliana*, SLOW ANION CHANNEL1 (SLAC1) represents the founder anion channel of a small gene family associated with CO₂ and abscisic acid-dependent stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). SLAC1, together with SLAC1 HOMOLOGOUS3 (SLAH3), represent the molecular determinants for S-type anion channel activity in guard cells (Geiger et al., 2009b, 2010, 2011), while in mesophyll cells, S-type anion channel activity appears to be driven by just SLAH3 (Geiger et al., 2009b, 2010, 2011; Demir et al., 2013). Both the SLAC1 and SLAH3 channels interact with distinct kinase-phosphatase pairs shown to be associated with water stress signaling (Geiger et al., 2009b, 2010, 2011; Brandt et al., 2012; Scherzer et al., 2012). Although anion channels of the SLAC/SLAH family show homology to tellurite resistance/C(4)-dicarboxylate transporters expressed in bacteria, archaea, and fungi, SLAC1 and SLAH3 exhibit a preference for nitrate and chloride but not for malate (Schmidt and Schroeder, 1994; Geiger et al., 2009b; Chen et al., 2010). These channels are further characterized by a NO₃⁻/Cl⁻ permeability ratio of ~10, in the case of SLAC1, and of 20 for SLAH3 (Geiger et al., 2009b, 2011).

Distant relatives of plant SLAC/SLAH anion channels from *Escherichia coli* have been annotated as tellurite resistance proteins (TehA) due to their association to the tehA/tehB operon (Walter et al., 1991; Taylor et al., 1994). While these early studies did not elucidate the function of the bacterial SLAC1 homolog, recently, Chen et al. (2010) determined the crystal structure of TehA from the bacteria *Haemophilus influenzae* (Hi-TehA). The structure of Hi-TehA (and the SLAC1 model derived from it) shows a trimeric assembly composed of quasisymmetrical subunits. Within the trimer, each monomer subunit consists of 10 transmembrane helices. The inner five-helix transmembrane ring contains a pore with a central, highly conserved, phenylalanine residue (in *Arabidopsis* SLAC1, F450) potentially representing the anion gate. Mutation of this pore phenylalanine turned Hi-TehA and its plant homolog, At-SLAC1, into an open anion channel (Chen et al., 2010). However, the molecular determinants of anion selectivity remain unclear.

Besides SLAC1, which is exclusively expressed in guard cells, four SLAC1 homologs (SLAH1 to 4) have been identified in *Arabidopsis* (Negi et al., 2008). Apart from SLAC1, SLAH3 is the only S-type anion channel that has been functionally characterized to date (Geiger et al., 2011; Demir et al., 2013). When expressed under the control of the SLAC1 promoter, SLAH1 and SLAH3 are able to rescue the *slac1-2* stomatal phenotype (Negi et al., 2008). By contrast, SLAH2, which is the closest homolog of SLAH3 (Dreyer et al., 2012), did not complement the phenotype caused by the loss of the chloride- and nitrate-permeable SLAC1 (Negi et al., 2008). This raises the question of whether SLAH2 exhibits different...
biophysical properties or interacting regulators than those identified in SLAH3 and SLAC1.

SLAH2 is expressed in the stele of the root, the cells that immediately surround the vasculature. These cells determine the anion composition of the sap flow between root and shoot (Köhler and Raschke, 2000; Köhler et al., 2002; Lin et al., 2008). Here, we used Xenopus laevis oocytes to study the anion selectivity of SLAH2 and compared it to that of SLAC1. In this way, we were able to establish SLAH2, in contrast to the NO₃⁻ and Cl⁻ permeable SLAC1, and homologs from other kingdoms, as an anion channel that transports nitrate exclusively (Chen and Hwang, 2008; Jentsch, 2008; Accardi and Picollo, 2009; Accardi and Picollo, 2010; Hedrich, 2012; Staub et al., 2012). Based on homology models for SLAC1 and SLAH2, structure-guided site-directed mutations were used to explore the molecular basis of the extraordinarily high nitrate selectivity of SLAH2. Exchanging the extracellular half of transmembrane helix (TM) 3 was sufficient to convert the chloride/nitrate permeable SLAC1 into a nitrate-selective channel similar to SLAH2. Within the pore region of SLAH2, the polarity of a single amino acid determined the distinctive nitrate selectivity of SLAH2, suggesting that the hydrophobicity difference between chloride and nitrate enables SLAH2 to differentiate between these two anions.

RESULTS

Stele-Specific Expression of SLAH2

To localize the site of SLAH2 expression, we isolated mRNA from various tissues of 6-week-old Arabidopsis Columbia-0 plants and found its transcripts restricted to the roots (Supplemental Figure 1A). Consistent with this, in plants expressing the SLAH2-promoter; GUS (β-glucuronidase) constructs, the root stele was identified as the SLAH2 expression site (Supplemental Figures 1B to 1E).

SLAH2 Physically Interacts with CPK21 and CIPK23 Protein Kinases

Based on this stele-specific expression, we tested whether SLAH2 exhibits stele-specific features that might conflict with those required for normal guard cell function. As a first step, we injected SLAH2 complementary RNA (cRNA) into Xenopus oocytes and used the two-electrode voltage clamp technique to analyze the biophysical properties of the channel. When expressed in Xenopus oocytes alone, SLAH2 was electrically silent. Since the guard cell anion channels SLAC1 and SLAH3 are activated by calcium-dependent protein kinases (CPKs) (Geiger et al., 2010, 2011; Brandt et al., 2012; Scherzer et al., 2012) and CBL-interacting protein kinase 23 (CIPK23) is required to activate plant K⁺ channels and nitrate transporters (Geiger et al., 2009a; Ho et al., 2009; Ho and Tsay, 2010), we performed BIFC (bimolecular fluorescence complementation)–based interaction assays in oocytes using these candidate protein kinases and SLAH2. SLAH2 was fused to the C-terminal half of yellow fluorescent protein (YFP), while the protein kinases of the CPK and CIPK types were fused with the complementary (N-terminal) half of the YFP molecule. When the fusion proteins were expressed alone, e.g., SLAH2:YFP_CT only, no YFP-specific fluorescence was observed in the oocytes (Supplemental Figure 2A). Upon co-injection of SLAH2:YFP_CT together with CPK21:YFP_NT, however, YFP fluorescence (BIFC) could be detected. Similar results were obtained with CIPK23:YFP_NT/CBL1 (Figures 1A and 1B; Supplemental Figures 2B and 2D). To rule out the possibility that the SLAH2-kinase interactions were overexpression artifacts, we performed experiments using CPK31, a close homolog of the calcium-dependent protein kinase 21, and CIPK24, a member of the CIPK family that activates the sodium/proton antiporter SOS1 (Qiu et al., 2002), as negative controls. Coexpression of CPK31:YFP_NT or CIPK24:YFP_NT with SLAH2:YFP_CT in oocytes resulted in no or only faint YFP fluorescence (Supplemental Figures 2C and 2E), indicating that the SLAH2-kinase interaction is specific.

Protein Kinase–Activated SLAH2 Mediates Nitrate Fluxes

To study the action of CPK21 on SLAH2 in an environment that was not limited by the oocyte’s resting cytosolic free Ca²⁺ concentration, we used truncated CPK variants (e.g., CPK21ΔEF) that lacked the junction and calcium binding domains. This modification renders CPK21 Ca²⁺ independent and, thus, constitutively active (Geiger et al., 2010, 2011). When SLAH2 was coexpressed with CPK21ΔEF, steady state currents of up to 10 µA appeared only in the presence of 30 mM NO₃⁻ in the external solution (Figure 1C). A similar result was obtained when coexpressing SLAH2 and CIPK23 together with the Ca²⁺ sensor protein CBL1 (Figure 1D).

To obtain a quantitative readout of the channel activation capacity of both protein kinases, we measured SLAH2-mediated currents at a membrane potential of −100 mV. When 10 ng SLAH2 cRNA was coinjected with 10 ng CPK21 or CIPK23 cRNA and 5 ng CBL1 cRNA, similar current amplitudes could be monitored for each kinase independently (Figure 1E). Coexpression of SLAH2 with CIPK23 in the absence of CBL1 did not result in SLAH2 activation. To test whether the SLAH2 channel properties depend on the nature of the activating kinase, we analyzed the voltage-dependent gating response of SLAH2 in the presence of nitrate. Following the expression of SLAH2 with either CPK21 or CIPK23/CBL, we analyzed the voltage-dependent open probability Po. The relative Po curves of the two kinases superimposed and thus support our hypothesis that both kinases activate SLAH2 in a similar way, as opposed to modifying the properties of the anion channel in a kinase-specific manner (Figure 1F).

SLAH2 Activation Requires Phosphorylation

The fact that SLAH2-derived anion currents were observed only when protein kinases were coexpressed suggested that phosphorylation might be necessary for channel activation. We therefore disrupted CPK21 and CIPK23 kinase activity by exchanging Asp-204 with Ala (CPK21ΔEFD204A) and Lys-60 with Asn (Harmon et al., 1994; Harper et al., 1994; Franz et al., 2011) and coexpressed SLAH2 alone or with either CPK21ΔEF, CPK21ΔEFD204A, CIPK23/CBL1, or CIPK23 K60N/CBL1. In this experiment, SLAH2-mediated anion currents were detected only in oocytes coexpressing SLAH2 with CPK21ΔEF or CIPK23/CBL1, but not in the absence of a kinase or with the kinase-inactive mutants (Figure 1E). To monitor phosphorylation of SLAH2 by CPK21,
we performed in vitro assays using recombinant CPK21 and the N-terminal cytoplasmic domain of SLAH2 (amino acids 1 to 142) as a substrate. Using radiolabeled [γ-32P]ATP in the presence of 2 μM free Ca2+, we observed autophosphorylation of CPK21 as well as transphosphorylation of the N terminus of SLAH2 (Supplemental Figure 3).

**SLAH2 Operates as Nitrate Channel**

Interestingly, in purely chloride-based media, the SLAH2-kinase pairs remained electrically silent (Figures 1C and 1D). In the presence of 10 mM NO3− at the external face, SLAH2 is activated (Figure 2A). Increasing the NO3− concentration in the bath 10-fold shifted the half-maximal activation voltage (V1/2) of SLAH2 from −80 to −180 mV (Figure 2B). This indicates that nitrate is acting as a permeating anion and channel opener. During 20-s voltage pulses to negative membrane potentials, SLAH2 currents activated rapidly and deactivated with slow kinetics (Figures 1C and 1D), a feature reminiscent of S-type anion channels (Schroeder and Keller, 1992; Marten et al., 2007). Consistent with the hypothesis of a nitrate-gated and -permeable conductance, SLAH2-mediated anion currents could be detected only in the presence of nitrate at the extracellular side. Upon reduction of the nitrate concentration, the reversal potential shifted to positive membrane voltages (Figure 2C). A 10-fold change in the external nitrate concentration resulted in a 58-mV shift of the reversal potential. To compare the permeability of SLAH2 to a range of anions, NO3− was replaced by HCO3−, malate, sulfate, iodide, bromide, and chloride (Figure 2D). The permeability sequence obtained was: NO3− (1) > iodide (0.688 ± 0.029) > bromide (0.090 ± 0.011) > chloride (0.012 ± 0.007) > malate (0.011 ± 0.001) > HCO3− (0.009 ± 0.002) > sulfate (0.006 ± 0.002). The mean nitrate to chloride permeability ratio [P(NO3−)/P(Cl−)] was 82.4. However, dependent on the quality of the respective oocyte batch P(NO3−)/P(Cl−) values of up to 200 were calculated. Thus, the root-expressed SLAH2 represents a nitrate channel that, considering the availability of physiologically relevant anions such as nitrate and chloride in the soil, is functionally permeable to nitrate only.

To characterize this extraordinarily strong preference for nitrate in more detail, we exposed SLAH2-expressing oocytes to different chloride/nitrate ratios (Figure 2E). Anion currents in the presence of 10 mM nitrate and 3 mM chloride in the bath medium were rather low. Increasing the chloride concentration to 90 mM affected neither the current amplitude nor the reversal potential or the relative Po; this further underpins our findings that chloride is not a SLAH2 substrate or channel modulator (Figures 2E and 2F). This characteristic clearly separates SLAH2 from the previously described S-type anion channels homologs, SLAC1 and SLAH3. Here, an increase in the chloride concentration in the presence of external nitrate shifted the reversal
Figure 2. NO$_3^-$ Dependence of SLAH2 Currents.

(A) Steady state currents (I$_{ss}$) of SLAH2- and CPK21ΔEF-coexpressing oocytes were normalized to the values at +40 mV in 100 mM NO$_3^-$ and plotted against the membrane potential. Currents were measured at the indicated voltages in the presence of the indicated concentrations of external NO$_3^-$ concentrations and 3 mM Cl$^-$ (n ≥ 5 experiments, mean ± SD).

(B) The relative open probability (rel. $P_o$) of SLAH2 at various NO$_3^-$ concentrations plotted against membrane potential. Data points were fitted with a single Boltzmann equation (solid lines, n ≥ 5 experiments, mean ± SD).

(C) Reversal potentials $V_{rev}$ of SLAH2- and CPK21ΔEF-expressing oocytes are shown as a function of the logarithmic external nitrate concentration. As expected for an anion-selective channel, the reversal potential shifted with a slope of 58 mV per decade to more negative values with increasing nitrate concentrations. To avoid loading of the oocytes with nitrate during the measurement, the reversal potentials were recorded in the current-clamp mode (n = 3 experiments, mean ± SD).

(D) Relative permeability (rel. permeability) of SLAH2 coexpressed with CPK21ΔEF in Xenopus oocytes (permeability for NO$_3^-$ was set to 1). Standard bath solution contained 50 mM of the indicated anion (pH 7.5). The reversal potentials used for the calculation of the relative permeability were recorded in the current-clamp mode (n = 4 experiments, mean ± SD).

(E) NO$_3^-$ and voltage dependence of steady state currents (I$_{ss}$) of SLAH2- and CPK21ΔEF-coexpressing oocytes. Currents were normalized to +40 mV in 100 mM NO$_3^-$ + 3 mM Cl$^-$ and plotted against the membrane potential. With 10 mM NO$_3^-$ in the bath medium SLAH2-mediated currents could be observed, which decreased at negative membrane potentials. The addition of 100 mM Cl$^-$ to the bath medium in the presence of 10 mM NO$_3^-$ had no
potential and the relative open probability to negative membrane potentials in both cases, indicating that SLAC1 as well as SLAH3 are permeable for chloride (Geiger et al., 2011). In the presence of 3 mM chloride in combination with 97 mM nitrate, the SLAH2 current amplitude was substantially higher than in the presence of elevated chloride concentrations (Figure 2E). Besides affecting the peak current, the 10-fold increase in nitrate concentration shifted the reversal potential to negative membrane potentials (Figure 2E, compared with Figure 2C). This feature characterized SLAH2 as an anion channel permeable to and gated by nitrate only.

Due to the strongly hyperpolarized membrane potential and the outward-directed anion gradients of plant cells, channel-mediated anion transport appears outwardly directed. Xenopus oocytes contain high concentrations of chloride as the major cytosolic anion. By contrast, nitrate does not represent a physiological anion in animal cells (Park et al., 2013). This raises the question about the nature of the charge carrier responsible for the inward currents (negative currents/anion efflux) observed in SLAH2-expressing oocytes. To substantiate that SLAH2 mediates the release of nitrate but not chloride from oocytes, we used a nitrate-preloading approach. At a holding potential of 0 mV, which is positive to the reversal potential of nitrate ($V_{\text{rev}}$), SLAH2-expressing oocytes were loaded with nitrate, while at −100 mV ($V_H < V_{\text{rev}}$) nitrate loading is thermodynamically not supported by the membrane potential. Following preloading at 0 mV, positive as well as negative currents could be recorded depending on the voltage applied (Figures 3A and 3C, compared with Figure 1C and 1D). When challenged with nitrate at −100 mV, however, inward currents (anion efflux) were not observed (Figures 3B and 3C). Note that inward currents at the tail voltage of −120 mV (third segment of the voltage protocol) were present only when membrane potentials positive of the $V_{\text{rev}}$ were applied within the preceding test voltage segment (second segment in the voltage protocol). This indicates that nitrate loading is required to evoke anion efflux. Thus, experiments employing oocytes preloaded with nitrate further support the notion that SLAH2 represents a nitrate-selective anion channel.

To simulate plant cell–like nitrate levels in a heterologous oocyte model, we injected 5 nmol 15N-nitrate into the oocytes (resulting in a final nitrate concentration of −10 mM). After a 5-min incubation in bathing solution containing 10 mM external nitrate, the amount of 15N-nitrate in the oocytes was determined by isotope ratio mass spectrometry. Noninjected control oocytes as well as oocytes expressing SLAH2, SLAC1, or CPK21 alone showed no release of the internal 15N-nitrate into the extracellular space within 5 min (Supplemental Figure 4A). Coexpression of SLAH2 or SLAC1 together with CPK2115EF resulted in a rapid efflux of 15N-nitrate, confirming that activation by protein kinases is essential for the transport activity of both anion channels. In the voltage clamp experiments described above (Figures 1C and 1D), we have shown that SLAH2-mediated currents depend on nitrate at the extracellular membrane face of oocytes. To test whether external NO3\(^{-}\) affects efflux of 15N-nitrate in a similar manner, the 15N-nitrate tracer pool in oocytes was followed in the absence or presence of 10 mM external nitrate in the bath solution (Supplemental Figure 4B). Whereas SLAH2-expressing oocytes maintained 50% of the 15N-nitrate load for 15 min in the absence of external NO3\(^{-}\), in SLAC1-expressing oocytes (which do not require external NO3\(^{-}\) for activity) release of 15N-nitrate under identical conditions was observed, with a 50% loss after just 5 min and >90% loss after 15 min. By contrast, in the presence of external nitrate, the efflux rate of SLAH2-expressing oocytes was similarly high as for oocytes expressing SLAC1. Although the outward-directed chemical gradient was at its maximum in the absence of external NO3\(^{-}\), SLAH2-mediated nitrate efflux was accelerated in the presence of external nitrate (Supplemental Figure 4B, compared with Figure 2A). The fact that 15N-nitrate injected, SLAH2-expressing oocytes did not transport nitrate, but required the presence of external nitrate for channel opening, indicates that the anion acts on the extracellular face of SLAH2 only.

**Homology Modeling of SLAC1 and SLAH2 to Reveal Potential Sites for Nitrate/Chloride Selection**

The crystal structure of Hi-TehA, a bacterial homolog of SLAC1, suggests that an inner five-helix transmembrane ring forms the pore with a central phenylalanine residue, which is invariant in this superfamily and seemingly blocks the pore (Chen et al., 2010; Dreyer et al., 2012). Whereas wild-type Hi-TehA showed no ion transport when expressed in oocytes, mutation of the pore phenylalanine (SLAC1 F450A) also gated SLAC1 (constitutively) open even in the absence of an activating kinase (Chen et al., 2010). However, the mutation did not markedly alter the anion selectivity profile of SLAC1. Thus, the molecular determinants of anion selectivity within the pore of SLAC/SLAH anion channels remained unknown.

Using two S-type anion channels (SLAC1 and SLAH2) that strongly differ in anion selectivity, we attempted to identify the pore forming residues involved in shaping the selectivity of the SLAC/SLAH-type channels. To first understand the potential constituents of the selectivity filter, 3D homology models of SLAC1 and SLAH2 were generated based on the crystal structure of Hi-TehA (Figure 4; PDB accession code 3M71; Chen et al., 2010).

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*Figure 2. (continued).*

Influence on the reversal potential ($V_{\text{rev}}$). By contrast, an increase in the external NO3\(^{-}\) concentration shifted the reversal potential to more negative voltages ($n = 4$ experiments, mean ± so). (f) Relative voltage-dependent open probabilities (rel. $P_o$) of SLAH2- and CPK21-mediated currents under conditions used in Figure 2E. Rel. $P_o$ was calculated from a −120-mV voltage pulse following the test pulses in the voltage range +60 to −200 mV in 20-mV decrements. Data points were fitted by a Boltzmann equation (continuous line; $n = 4$ experiments, mean ± so).
As both 3D models were built from the same template and because of the high sequence similarity between SLAC1 and SLAH2, with no insertion or deletions inside the pore region, both homology models of SLAC1 and SLAH2 (Supplemental Figure 5; Figures 4A and 4B) share highly similar pore geometries with a diameter of 5 to 6 Å. The conserved side chain of the gating phenylalanines, Phe-450 or Phe-402, are located in the middle of the pore, as described by Chen et al. (2010) for Hi-TehA and SLAC1. A detailed comparison of the pore regions, however, revealed differences between SLAC1 and both SLAH2 and Hi-TehA. Besides the conserved gating phenylalanine, two residues above the phenylalanine in the outward facing vestibule are conserved between SLAC1 (Phe-276 and Trp-404) and SLAH2 (Phe-231 Trp-356) and may contribute to pore blockage as well as rendering the SLAC1/SLAH2 vestibule on the extracellular face shallower than that of Hi-TehA (Supplemental Figure 6). By contrast, the geometry and size of the intracellular facing cavities of SLAC1 and SLAH2 and the bacterial homolog Hi-TehA are quite similar. Comparing the three channel proteins, most of the 29 pore-lining residues are either aliphatic or aromatic, giving the pore a hydrophobic character (Supplemental Figure 5).

Whole-oocyte currents of oocytes coexpressing SLAH2, CIPK23, and CBL1 in standard medium containing 30 mM NO$_3^-$ The experiment was performed at two different holding potentials using the standard voltage protocol. (A) When the oocytes were clamped to 0 mV, single voltage pulses were applied starting from +60 to −200 mV in 20-mV decrements. Representative cells are shown. (B) When the oocytes were clamped to −100 mV, the voltage pulses increased from −200 to +60 mV in 20-mV steps. Representative cells are shown. (C) Steady state currents ($I_{SS}$) of SLAH2 and CIPK23/CBL1 coexpressing oocytes measured as in (A) ($V_{hold} = 0$ mV pos > neg) or (B) ($V_{hold} = −100$ mV neg > pos) (*$n = 5$ experiments, mean ± so).
restrained by the replacement of amino acids with a similar size or only slightly different biochemical character.

**Pore-Lining Phenylalanine Residues Are Involved in Nitrate Selectivity of SLAH2**

To examine whether Phe-402 in SLAH2 exhibits similar gating properties as Phe-450 in SLAC1 (Supplemental Figure 7B compared with Chen et al. [2010]) and Phe-262 in Hi-TehA, we replaced this residue with alanine and expressed the mutant protein in *Xenopus* oocytes. The mutant SLAH2 F402A indeed had an open channel that no longer required a protein kinase for anion channel activity (Supplemental Figure 7A compared with Chen et al. [2010]). Expression of SLAH2 F402A alone displayed macroscopic anion currents similar to SLAH2 wild-type channel activated by CIPK23/CBL1 (Figure 5A), but nitrate was nevertheless required in the extracellular bath solution for ion conduction (Supplemental Figure 7A).

In the homology models, another phenylalanine located on transmembrane helix (TM) 3 in the SLAH2 and SLAC1 pore is in close proximity to the gating Phe (Figures 4A and 4B; Supplemental Figures 6A and 6B). Together with the gating Phe residue, Phe-231 of SLAH2 and Phe-276 of SLAC1 might also serve to constrict the pore and may have to also be displaced in order to establish an anion conducting state (Figures 4A and 4B; Supplemental Figures 6A and 6B). Mutation of Phe-231 alone to Ala in SLAH2 did not result in a per se F402A-like open channel. When SLAH2 F231A was coexpressed with CIPK23/CBL1, however, macroscopic chloride currents similar to wild-type SLAC1 could be monitored in the absence of external nitrate (Supplemental Figure 7C compared with Figure 5B). To better validate the permeability of wild-type SLAH2 and mutants thereof for different anions, the chord conductance at +40 mV and −120 mV was calculated. This measure is directly proportional to the permeability of a channel for a specific anion. When we compared SLAH2 F231A to wild-type SLAH2, the chloride chord conductance was markedly increased and reached 50% (at −120 and +40 mV) of the SLAC1 wild-type chord conductance for chloride (Supplemental Figure 7E). This indicates that replacement of Phe-231 with alanine converts the nitrate-specific SLAH2 into a chloride-permeable SLAC1-like anion channel (Supplemental Figure 7E). Mutation of the respective Phe in SLAC1 to Ala (F276A) also resulted in enhanced chloride permeability relative to nitrate (Supplemental Figures 7D and 7E). These findings show that the replacement of Phe-231 with Ala in the SLAH2 pore results in a SLAC1-like anion channel.

**Figure 5.** Mutations in TM3 Influence Ion Selectivity of SLAC1 and SLAH2.

Instantaneous currents ($I_{\text{inst}}$) of *Xenopus* oocytes in standard buffers containing different anions (gluconate, chloride, or nitrate) are plotted against the applied voltage.

(A) and (C) SLAH2 wild type and the mutant SLAH2C1(271-281).

(B) and (D) SLAC1 wild type and the corresponding mutant. The channel mutants and kinases expressed in the oocytes are indicated in the figure ($n = 3$ experiments, mean ± s.e.).
permeability with broader anion specificity. The latter SLAH2 mutation was able to affect channel opening independently from the presence of extracellular nitrate. Thus, the molecular basis for SLAH2 nitrate gating and selectivity appears to be associated with the pore of SLAH2.

**The C-Terminal Half of TM3 Determines S-Type Channel Selectivity**

Since both phenylalanine residues are conserved between SLAC1 and SLAH2, it is unlikely that these amino acids residues could directly exert the different anion selectivity. Analysis of the 3D models suggests that the side chains of the critical phenylalanines, Phe-450 and Phe-276 in SLAC1, and Phe-402 and Phe-231 in SLAH2, have to be displaced to allow anion permeation, as has also been proposed for Phe-450 in SLAC1 and Phe-262 in HiTehA (Chen et al., 2010). Due to packing, side chain displacement is restricted by neighboring pore-lining residues and the helical backbone of the five inner TMs (Figures 6A to 6C; Supplemental Figures 6A and 6B). When comparing the environment of the critical phenylalanines in SLAC1 and SLAH2, the most prominent differences were found in the extracellular half of TM3 (residues 226 to 236 in SLAH2 and residues 271 to 281 in SLAC1; Figures 6A to 6C; Supplemental Figure 5). To study the effect of these residues, we exchanged corresponding sites between SLAH2 and SLAC1 and expressed the TM3 chimeras in Xenopus oocytes. The SLAC1 chimera containing amino acids 226 to 236 from SLAH2 was named SLAC1 H2(226-236) and the respective SLAH2 version SLAH2 H2(226-236) (Supplemental Figure 8A). This result was comparable to the results obtained for SLAH2 either (Figure 6F). Another difference was observed at the exchange of the C-terminal half of TM3, which faces the extracellular side, was sufficient to convert the guard cell channel SLAC1 into a SLAH2-like, strict nitrate-selective anion channel. Although Phe-231 and Phe-402 in SLAH2 and Phe-276 and Phe-450 in SLAC1 are in close proximity in the 3D models, with the shortest distance between the two aromatic rings measures ~3.4 Å, which would be too small to let anions pass. A possible mechanism for allowing anion conduction would require a rotation or flip of the aromatic rings along the \( \chi^2 \)-torsion angle (Figures 6A to 6C). When focusing on the C-terminal half of TM3, which altered the properties of the channels, three residues were identified from the models that might influence the flexibility of Phe-231 in SLAH2 and Phe-276 in SLAC1. In SLAC1, a bulky methionine residue, Met-275, presses against Phe-276, which together with residues on the TM3 located one turn up or down, Val-272 and Ile-279, could potentially restrict conformational rearrangement of the phenylalanine side chain. In the model of SLAH2, a similar smaller leucine residue, Leu-230, packs against Phe-231 (Figures 6A to 6C). Thus, based on the homology modeling, the different packing of the Phe-231 side chain in SLAH2 compared with its counterpart Phe-276 in SLAC1 could possibly present the molecular cause for the distinct selectivity characteristics of both anion channels. To test this hypothesis, we exchanged Met-275 to Leu in SLAC1 (M275L) and Leu-230 of SLAH2 with Met (L230M). Additionally, we created a triple mutant in which we exchanged all neighboring residues in SLAC1 and SLAH2 mentioned above that might have an influence on the conformational freedom of Phe-231 in SLAH2 and Phe-276 in SLAC1 (SLAH2 I227V L230M L234I and SLAC1 V272I L279L I282V). However, neither the single nor the triple mutants showed any differences in their selectivity compared with the respective wild-type channels (Supplemental Figure 9).

In search of further differences in the C-terminal half of TM3 that could alter the chemistry at this site, we found that SLAC1 harbors a serine at position 280 in close proximity to the pore while SLAH2 has a glycine at the respective position (Gly-235). The exchange of the polar Ser-280 to a nonpolar glycine in SLAC1, however, did not change the selectivity of the guard cell anion channel (Figure 6F). Similarly, the respective mutation in SLAH2 (G235S) did not change the nitrate-selective character of SLAH2 either (Figure 6F). Another difference was observed at
Figure 6. Structural Differences of Transmembrane Helix 3 Determine the Selectivity Differences between SLAC1 and SLAH2.

(A) to (C) Structural differences in the pore region of SLAC1 and SLAH2. Close-up view of TM3 of SLAC1 (A), an overlay of SLAC1 and SLAH2 (B), and SLAH2 (C) with the side chains and TM4 of SLAC1 (carbon atoms in cyan) and SLAH2 (carbon atoms in magenta). Pore lining residues are facing left. The position of pore-blocking Phe-450 and Phe-402 on TM9 is shown.

(D) and (E) Instantaneous currents ($I_{\text{inst}}$) of Xenopus oocytes coexpressing the mutants SLAH2 S228V (C) or SLAC1 V273S (D) with CIPK23/CBL1 in standard buffers containing different anions (glucionate, chloride, or nitrate) are plotted against the applied voltage ($n = 4$ experiments, mean ± se).

(F) Chord conductance at +40 and −120 mV in standard solutions containing 100 mM Cl⁻. The chord conductance was calculated from the instantaneous currents in Cl⁻ as well as in NO₃⁻ containing buffers from oocytes coexpressing SLAH2 or SLAC1 wild type, or the different channel mutants, with CIPK23 and CBL1. The chord conductance was normalized to 100 mM NO₃⁻ (dashed line) ($n = 3$ experiments, mean ± se).
position 228 in SLAH2 corresponding to residue 273 in SLAC1. While SLAH2 is equipped with a polar serine (Ser-228), SLAC1 carries a nonpolar valine (Val-273) at the respective position. Interestingly, this single substitution of Ser-228 by valine was sufficient to convert SLAH2 into a SLAC1-like, chloride- and nitrate-permeable anion channel indicated by macroscopic anion currents in nitrate and chloride solutions (Figure 6D). The chord conductance for chloride of SLAH2 S228V increased to 60% of the observed NO$_3^-$ conductance (Figure 6F). Thus, at least in SLAH2, anion selectivity seems to be encoded locally by a single residue, which is in close proximity to the gating phenylalanines. However, performing the reverse amino acid exchange by substituting Val-273 in SLAC1 to the respective serine residue as in SLAH2 did not convert SLAC1 into a NO$_3^-$-selective channel (Figures 6E and 6F). Thus, although a single mutation in SLAH2 was sufficient to yield an anion channel with SLAC1-like properties, in SLAC1 additional elements besides the nature of the amino acid at position 273 are involved in exerting anion selection, possibly indicating that the pore geometry (despite the high sequence similarity between SLAC1 and SLAH2) does slightly differ.

**DISCUSSION**

How does one build and operate an anion-selective channel? Our knowledge concerning the selectivity of animal anion channels of the CLC type is well advanced (Dutzler et al., 2003). X-ray structures of two prokaryotic CLC Cl$^-$ channel homologs from *Salmonella enterica serovar typhimurium* and *Escherichia coli* at 3.0 and 3.5 Å, respectively (Dutzler et al., 2002), revealed a homodimeric membrane protein. Each subunit forms its own independent anion selectivity filter. Each anion binding site stabilizes a single chloride ion at three sites within the highly constricted permeation pathway (Dutzler et al., 2003). Together with specific neighboring residues, these binding sites determine the selectivity of bacterial CLCs. CLC channels are gated by the side chain carboxylate group of a central highly conserved glutamate that closes the pore by mimicking a chloride ion. The anion selectivity with the order chloride > bromide > nitrate > iodide found for animal CLC channels confirms the existence of a high-field-strength anion binding site (Wright and Diamond, 1977; Picollo et al., 2009).

*Arabidopsis* contains seven homologs, named CLC-a to CLC-g, which all potentially reside in intracellular membranes (Lv et al., 2009). The most thoroughly investigated plant CLC homolog is CLC-a, which, like the bacterial counterpart, utilizes the pH gradient across the vacuolar membrane to transport nitrate into the organelle (De Angeli et al., 2006; Bergsdorf et al., 2009). The high nitrate selectivity at its cytosolic side is consistent with the nature of the amino acid at position 273 are involved in ion selectivity, possibly indicating that the pore geometry (despite the high sequence similarity between SLAC1 and SLAH2) does slightly differ.

The structure of the bacterial Hi-TehA reported by Chen et al. (2010) provided a molecular basis to analyze ion transport and selectivity for members of this class of anion channel proteins, which exhibit architecture clearly distinct from those of other known animal channels such as the CLCs, CFTR, or TMEM16A (Dutzler et al., 2002; Gadsby et al., 2006; Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Zifarelli and Pusch, 2010). This structure guided our functional analysis of the molecular nature of the SLAC/SLAH selectivity filter.

Replacing the gating phenylalanine (Phe-402 in SLAH2 and Phe-450 in SLAC1) in the pore of SLAC1 and SLAH2 by alanine rendered both anion channels open in a kinase-independent fashion (Supplemental Figures 7A and 7B; compared with Chen et al., 2010). However, these mutants did not display markedly altered anion selectivity compared with the corresponding wild type (Figures 5A and 5B). The homology models of SLAH2 and SLAC1 highlighted a second phenylalanine (Phe-231 in SLAH2, Phe-276 in SLAC1) located on TM3 and in close proximity to Phe-402 or Phe-450 on TM9 of SLAH2 and SLAC1, respectively. Both phenylalanine residues on TM3 and TM9 together seem to constrict the pore. According to Chen et al. (2010), the side chain of the gating phenylalanine (Phe-450 in SLAC1 and Phe-402 in SLAH2) is present in a high-energy conformation and has to be displaced to unblock the pore. Because Phe-231 potentially restricts the mobility of Phe-450 (and vice versa), it is tempting to speculate that the side chain conformations of both phenylalanines rearrange in a concerted manner upon ion transport. Despite the fact that mutation of the Phe-402 in SLAH2 rendered the channel constitutively open, the SLAH2 mutant F231A still required coexpression of an activating kinase to convert SLAH2 into a SLAC1-like, chloride- and nitrate-permeable anion channel indicated by macroscopic anion currents in nitrate and chloride solutions (Figure 6D). The chord conductance for chloride of SLAH2 S228V increased to 60% of the observed NO$_3^-$ conductance (Figure 6F). Thus, at least in SLAH2, anion selectivity seems to be encoded locally by a single residue, which is in close proximity to the gating phenylalanines. However, performing the reverse amino acid exchange by substituting Val-273 in SLAC1 to the respective serine residue as in SLAH2 did not convert SLAC1 into a NO$_3^-$-selective channel (Figures 6E and 6F). Thus, although a single mutation in SLAH2 was sufficient to yield an anion channel with SLAC1-like properties, in SLAC1 additional elements besides the nature of the amino acid at position 273 are involved in exerting anion selection, possibly indicating that the pore geometry (despite the high sequence similarity between SLAC1 and SLAH2) does slightly differ.
facilitating $\text{Cl}^-$-activated $\text{Cl}^-$ transport (Pusch et al., 1995; Chen and Miller, 1996). Upon chloride conductance, chloride ions positively modulate the open probability of the CLC channel gate. Here, a glutamate side chain gates the pore open upon binding to a chloride ion within the selectivity filter of CLC channels (Dutzler et al., 2002). Thus, selective conduction and gating is coupled within the pore.

In the vicinity of Phe-402/450 of SLAH2 and SLAC1, we uncovered prominent differences in the extracellular half of TM3 (Figures 6A to 6C; Supplemental Figure 5). Exchanging this stretch of amino acids between SLAH2 and SLAC1 converted SLAC1 into a nitrate-selective anion channel with similar ion selectivity and transport capabilities as wild-type SLAH2. Vice versa, SLAH2 equipped with amino acid residues 271 to 281 from SLAC1 provided SLAH2 with SLAC1-like anion channel properties; however, a complete conversion of SLAH2 into a chloride- and nitrate-permeable channel like SLAC1 could not be obtained. This indicates that although the C-terminal half of TM3 plays a major role as selectivity filter, it is not fully responsible for determining the ion selection. Further inspection of the 3D homology models of SLAH2 and SLAC1 highlighted the polar serine residue at position 228 in SLAH2 within the C-terminal half of TM3. When we exchanged Ser-228 for valine, which is the respective amino acid in SLAC1, SLAH2 gained SLAC1-like chloride permeability and channel activation became independent of extracellular nitrate (Figures 6D and 6F). In addition, the movement of the side chain Phe-231 in SLAH2 might be restricted by residues present in the C-terminal half of TM3 in such a way that nitrate, but not chloride, can pass through the anion channel pore. Thus, the peculiar selectivity of SLAH2 might originate from the facilitated coordination of nitrate but not chloride at the polar serine residue 228 and may influence the concerted mobility or conformational rearrangements of the gating Phe-402 and Phe-231.

**SLAH2 Probably Evolved to Facilitate Root-Shoot Nitrate Transport**

Our findings suggest that similar to SLAH3 and SLAC1, the interaction of SLAH2 with distinct protein kinases (compared with Scherzer et al., 2012) renders the stele-expressed anion transporter active. We established that SLAH2 represents a nitrate-selective channel, demonstrating that such channels exist in living systems. Its strict nitrate specificity distinguishes SLAH2 from SLAC1 as well as from SLAH3; therefore, SLAH2 expressed in the SLAC1 knockout mutant might not be capable of recovering the chloride release required for stomatal closure (Negi et al., 2008). Given this peculiar selectivity among the SLAC/SLAH family members, the strict nitrate selectivity is of no advantage to guard cells located at the end of the transpiration stream, but would be highly advantageous in the root, where water and nutrients enter the transpiration stream and where the plant obtains soil nitrate and at times is exposed to high NaCl load under salt stress. The SLAH2-type anion channels in roots, with a strict NO$_3^-$ selectivity, would facilitate the preferential transport of nitrate, which is a major source of nitrogen for plants. Future physiological and electrophysiological studies with SLAH2 loss-of-function mutants are needed to elucidate whether these hypothesized physiological functions indeed explain the evolution of this NO$_3^-$-specific channel, SLAH2, in plants.

**METHODS**

**Detection of SLAH2 Transcripts by Quantitative RT-PCR**

Quantification of ACTIN2 and 8 as well as SLAH2 transcripts was performed by real-time PCR as described elsewhere (Ivashikina et al., 2005). The different tissues of 6- to 8-week-old Arabidopsis thaliana ecotype Columbia (Col-0) plants were harvested for RT-PCR analysis. Following RNA extraction and cDNA synthesis, quantitative PCR experiments were performed as described by Ivashikina et al. (2005). Transcripts were each normalized to 10,000 molecules of ACTIN2/8. Primer sequences are provided in Supplemental Table 1.

**Cloning and cRNA Generation**

The cDNAs of SLAH2, SLAC1, CPK21/31, CIPK23/24, and CBL1/4 were cloned into oocyte (BIFC) expression vectors (based on pGEM vectors, see Supplemental Table 2) by an advanced uracil-excision-based cloning technique as described (Nour-Eldin et al., 2006). Site-directed mutations were introduced by means of a modified USER fusion method as described by Dadacz-Narloch et al. (2011). Primer sequences are provided in Supplemental Table 1. For functional analysis, cRNA was prepared with the mMESSAGE mMACHINE T7 transcription kit (Ambion). Oocyte preparation and cRNA injection were performed as described (Becker et al., 1998). For oocyte BIFC and electrophysiological experiments, 10 ng of SLAH2, SLAC1, or CPK cRNA and 2 ng CIPK or CBL cRNA were injected.

**Oocyte Recordings**

In double-electrode voltage clamp studies, oocytes were perfused with Tris/MES buffers. The standard solution contained 10 mM Tris/MES (pH 5.6), 1 mM Ca(gluconate)$_2$, 1 mM Mg(gluconate)$_2$, 100 mM NaNO$_3$, and 1 mM LaCl$_3$. To balance the ionic strength, we compensated for the NO$_3^-$ or Cl$^-$ variations with gluconate. Solutions for anion selectivity measurements were composed of 50 mM Cl$^-$, HCO$_3^-$, SO$_4^{2-}$, NO$_3^-$, gluconate$^-$, or malate$^-$ sodium salts; 1 mM Ca(gluconate)$_2$; 1 mM Mg(gluconate)$_2$; and 10 mM Tris/MES (pH 7.5). Osmolality was adjusted to 220 mosmol/kg with D-sorbitol. Standard voltage protocol was as follows: Starting from a holding potential ($V_h$) of 0 mV, single-voltage pulses were applied in 20-mV decrements from +60 to −200 mV. Steady state currents ($I_{ss}$) were extracted at the end of the 20-s test pulses. The relative open probability $P_0$ was determined from current responses to a constant voltage pulse to −120 mV subsequent to the test pulses. These currents were normalized to the saturation value of the calculated Boltzmann distribution. The half-maximal activation potential ($V_{1/2}$) and the apparent gating charge ($\alpha$) were determined by fitting the experimental data points with a single Boltzmann equation. To avoid nitrate loading of the oocytes (Figure 3B), a holding potential of −100 mV was used in combination with a modified standard voltage protocol (20-mV steps from −200 to +60 mV).

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Instantaneous currents ($I_{int}$) were extracted right after the voltage jump from the holding potential of 0 mV to 50-ms test pulses ranging from +70 to −150 mV. To calculate the chord conductance, the reversal potential ($V_{rev}$) was determined by fitting the experimental data points with a single Boltzmann equation. To avoid nitrate loading of the oocytes (Figure 3B), a holding potential of −100 mV was used in combination with a modified standard voltage protocol (20-mV steps from −200 to +60 mV).

Using the instantaneous currents at $V_{rev}$, the chord conductance ($G_{chord}$) was calculated with the equation $G_{chord} = I_{int}/(V − V_{rev})$, as described previously (Hodgkin...
and Huxley, 1952; Hodgkin et al., 1952). To avoid loading of oocytes with nitrate and to keep the cytosolic anion composition constant for correct reversal potential determination in response to nitrate concentration changes (Figure 2C) in the external medium or during anion selectivity measurements (Figure 2D), the reversal potentials were recorded in the current-clamp modus.

**BiFC Experiments**

For documentation of the oocyte BiFC results, images were taken with a confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss Jena) equipped with a Zeiss Plan-Neocon 40×/0.5 objective. Images were identically processed (low-pass filtered and sharpened) with the image-acquisition software LSM 5 Pascal (Carl Zeiss).

**Protein Purification and in Vitro Kinase Assays**

The coding sequence for CPK21, as well as those for the SLAH2 N- and C-terminal domains, were cloned into the USER-compatible expression vector pGEX 6P1-USER and transformed into Escherichia coli (DE3) pLYS3 strain (Novagen). Bacteria were grown to an OD of 0.5 to 0.8 at 600 nm, and production of glutathione S-transferase fusion proteins was induced by 0.4 mM isopropylthio-

**Quantification of 15N Abundance and Total Nitrogen Concentration**

*Xenopus laevis* oocytes were injected with 5 nmol 15N-labeled NO3− per oocyte in the absence of external nitrate. Injected oocytes were incubated in standard buffers containing 0 or 10 mM nitrate. At different time points, single oocytes were transferred to tin capsules and oven-dried overnight at 80°C. Total nitrogen concentrations and 15N abundances were determined using an elemental analyzer (NC2500; CE Instruments), coupled to a Finnigan MAT Delta Plus Isotope Ratio Mass Spectrometer (Delta Plus; Thermo Finnigan MAT). Working standards (glutamic acid) were analyzed after every twelfth sample to detect a potential instrument drift over time. Standards were calibrated against the primary standard USGS 41 (8.57% 15N; 47,600) for δ15N.

**Supplemental Data**

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

**Supplemental Figure 1. Localization of SLAH2 in Arabidopsis thaliana.**
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AUTHOR CONTRIBUTIONS


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A Single-Pore Residue Renders the Arabidopsis Root Anion Channel SLAH2 Highly Nitrate Selective
Tobias Maierhofer, Christof Lind, Stefanie Hüttl, Sönke Scherzer, Melanie Papenfuß, Judy Simon, Khaled A.S. Al-Rasheid, Peter Ache, Heinz Rennenberg, Rainer Hedrich, Thomas D. Müller and Dietmar Geiger

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