

Investigation of the regulation mechanism of *Arabidopsis thaliana* anion channel SLAH2

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Abstract: In recent years a slow-type anion channel (SLAC) family composing five members, including slow anion channel associated 1 (SLAC1) and four SLAC1 homologs (SLAHs), has been identified in *Arabidopsis thaliana*. The anion channels are activated by calcium-dependent protein kinases (CPKs) and play crucial roles in mediating anion fluxes across plasma membranes. SLAH2 is specifically expressed in root stele cells and has been characterized as a nitrate-selective anion channel activated by CPK21 and CBL-interacting protein kinase 23 (CIPK23). However, the interaction between CPKs and SLAH2 and how it is regulated remains to be elucidated. In this study we found that SLAH2 could be activated by a few CPKs from three subgroups of the CPK family. Out of those CPKs, the constitutively active form of CPK2 from subgroup 1 activates SLAH2, whereas the native as well as constitutively active form of CPK3 from subgroup 2 and CPK7, CPK10, and CPK32 from subgroup 3 activate SLAH2. We also explored the role of SLAH2 in root-to-shoot nitrate transport by measuring the nitrate contents in xylem sap, but no significant differences were observed between the *slah2-1* T-DNA insertion mutant and *SLAH2* overexpression lines. Together, these data demonstrate that SLAH2 is regulated by multiple CPKs with variable Ca²⁺ sensitivities. Further research is required to understand the role of SLAH2 in nitrate transport.

Key words: *Arabidopsis*, anion channel, SLAH2, CPKs, nitrate, calcium

1. Introduction

Anion channels in the plasma membrane of plant cells play essential roles in anion nutrient intake from soil and long-distance transport in vivo in higher plants. In recent years, a slow-type (S type) anion channel family including SLAC1 and four SLAC1 homologs (SLAHs), namely SLAH1–SLAH4, was reported in *Arabidopsis thaliana* (Negi et al., 2008; Vahisalu et al., 2008). All the members of the SLAC1/SLAHs family are localized in the plasma membrane of plant cells and SLAC1 is specifically expressed in guard cells (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009, 2011; Lee et al., 2009; Maierhofer et al., 2014). The activation of SLAC1 causes anion efflux from guard cells and further leads to stomatal closure (Vahisalu et al., 2008). Accordingly, *slac1* loss-of-function mutants showed impaired stomatal closure phenotypes in response to diverse stimuli, including ABA, high CO₂, and ozone (Negi et al., 2008; Vahisalu et al., 2008). SLAH3 has an overlapping function with SLAC1 in guard cells for stomatal closure, but it exhibits a stronger selectivity for nitrate over chloride compared with SLAC1 (Geiger et al.,

2011). On the other hand, SLAH3 is also highly expressed in roots, mesophyll cells, and pollen tubes (Geiger et al., 2009; Gutermuth et al., 2013; Zheng et al., 2015). SLAH3 is regulated by CPK2 and CPK20 in a Ca²⁺-dependent manner and is involved in the regulation of pollen tube growth (Gutermuth et al., 2013). SLAH3 also plays a role in nitrate-dependent alleviation of ammonium toxicity in *Arabidopsis* roots (Zheng et al., 2015). Moreover, SLAH1 and SLAH3 are involved in chloride (Cl⁻) translocation from root to shoot (Cubero-Font et al., 2016; Qiu et al., 2016). SLAH4 is also expressed in roots (Zheng et al., 2015), but its functional characterization has not been reported yet. SLAH2, a close homolog of SLAH3, is expressed in the root stele cells, which determine the anion composition of the xylem sap. SLAH2 is strictly permeable to nitrate (Maierhofer et al., 2014), and this ion selectivity of SLAH2 is obviously different from that of SLAC1 (Geiger et al., 2009; Lee et al., 2009).

Nitrate absorbed from soil by roots needs to be loaded into the root xylem vessel and is further transported to aerial parts of the plants for assimilation. Three nitrate

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transporters from the NRT1 family, NRT1.5, NRT1.8, and NRT1.9, are involved in nitrate transport between roots and shoots (Lin et al., 2008; Li et al., 2010; Wang and Tsay, 2011). Considering the root-specific expression pattern and strict nitrate selectivity, SLAH2 is speculated to be involved in nitrate transport in plants (Maierhofer et al., 2014). However, no experimental evidence is available to validate this hypothesis.

A typical CPK has four distinct domains: a variable N-terminal domain, a serine/threonine protein kinase domain, an autoinhibitory domain, and a C-terminal calmodulin-like domain, which has four Ca²⁺-binding EF hand motifs (Cheng et al., 2002). The autoinhibitory domain is involved in the inhibition of their kinase activity in the absence of Ca²⁺. The binding of Ca²⁺ to the EF hand motifs results in intramolecular rearrangements and releases their kinase activity from the autoinhibition. The *Arabidopsis* genome contains 34 CPKs, which are divided into four subgroups (Cheng et al., 2002). Fourteen CPK members are expressed in roots, and 3 representative subgroups have been investigated for their Ca²⁺-dependence and -sensitivities (Boudsocq et al., 2012). The CPKs showed highly variable Ca²⁺-dependence; most of the CPKs from subgroups 1 and 2 were Ca²⁺ sensitive, whereas most of the CPKs from subgroup 3 showed low sensitivity to Ca²⁺, while a few other members from subgroup 3 were even insensitive to Ca²⁺ (Boudsocq et al., 2012).

The activity of SLAC1 and SLAHs is regulated by protein kinases via phosphorylation. Multiple CPKs are able to activate SLAC1 and SLAH3 in guard cells for stomatal closure. CPK3 is expressed in both guard cells and mesophyll cells, and it is involved in ABA- and Ca²⁺ activation of slow-type anion channels in guard cells (Mori et al., 2006; Scherzer et al., 2012). Salt stress triggers the activation of CPK3, which in turn phosphorylates the *Arabidopsis* vacuolar K⁺ channel TPK, which is essential for salt stress adaptation in *Arabidopsis*. CPK3 is also required for MAPK-independent salt-stress acclimation in *Arabidopsis* (Mehlmer et al., 2010; Latz et al., 2013).

A recent study showed that SLAH2 was able to specifically interact with and be activated by CPK21 and CIPK23 (Maierhofer et al., 2014). Therefore, SLAH2 could be activated and regulated by multiple CPKs in roots, similar to the activation of SLAC1 and SLAH3 by multiple CPKs in guard cells. However, no experimental evidence regarding the activation and regulation of SLAH2 by multiple CPKs has been reported yet.

In this research we identified a few root-expressed CPKs that activate SLAH2. We also investigated the possible role of SLAH2 in nitrate transport. However, no clear difference in xylem sap nitrate contents was observed between the *slah2-1* mutant, SLAH2 overexpression lines, and wild-type plants.

2. Materials and methods

2.1. Plant materials and growth conditions

For the growth of *Arabidopsis thaliana* plants (Columbia ecotype), seeds of wild-type, *slah2-1* mutant, and SLAH2 overexpression lines were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) medium containing 1% (w/v) sucrose and 0.8% (w/v) agar for 5 days. Seedlings were transplanted to hydroponic solution for 4 weeks in a controlled growth room under a 16-h-light/8-h-dark cycle at a photon fluence rate of approximately 75 μmol m⁻² s⁻¹ during daytime at a temperature of 21 ± 0.5 °C as previously described (Zhang et al., 2016).

2.2. Plant transformation

The full-length coding sequence of SLAH2 was cloned into the binary vector pCAMBIA1300S under a 35S promoter (see Table S1 for primers). The construct pBCAM1300S-35S::SLAH2 was mobilized into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* wild-type and *slah2-1* mutant plants were transformed using the floral dip method (Clough and Bent, 1998) to generate overexpression lines and complementation lines, respectively. Seeds from T0 plants were screened on MS medium containing 50 mg L⁻¹ hygromycin. Homozygous lines from the T3 generation were selected and used for experiments.

2.3. Quantitative real-time PCR (qRT-PCR)

To determine SLAH2's expression in plants, total RNA was extracted from 3-week-old *Arabidopsis* plants using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from DNaseI-digested total RNA using M-MLV reverse transcriptase (Promega). qRT-PCR was performed using TransStart Tip Green qPCR Super Mix (TransGen Biotech) on a Bio-Rad CFX Connect Real-Time System according to the manufacturer's protocol (Zhang et al., 2016). 18S ribosomal RNA was used as a reference for normalization of the gene expression. Primers for qRT-PCR are listed in Table S1.

2.4. Voltage-clamp recordings in *Xenopus* oocytes

The coding sequences of AtSLAH2, AtCPKs, and the truncated versions of AtCPKs, with their C-terminus-harboring autoinhibitory domains and the EF hands (Ca²⁺-binding sites) deleted, were cloned into the pGEMHE vector as described (Brandt et al., 2012; Zhang et al., 2016) (See Table S1 for primers). The truncated versions of AtCPKs were called AtCPKDEFs. The truncated versions of CPK2, CPK3, CPK4, CPK7, CPK10, CPK11, and CPK32 refer to Met1-Val444, Met1-Ile336, Met1-Ile283, Met1-Ile337, Met1-Ile321, Met1-Ile284, and Met1-Leu321 peptides, respectively. The cRNAs were prepared in vitro using the T7 RiboMAX™ large-scale RNA production system (Promega). The oocytes were isolated from *Xenopus laevis* and injected with the cRNA

as indicated. Oocytes injected with water were used as a negative control. All the coexpressing mixtures were injected into oocytes with the amount of 6 ng each in a total volume of 50 nL. The injected oocytes were incubated at 16 °C in a modified bath solution supplemented with gentamycin (0.1 mg/mL) for 2–3 days (Zhang et al., 2016). For anion channel current recordings, the bath solution contained 1 mM Ca-gluconate, 1 mM Mg-gluconate, 1 mM K-gluconate, 50 mM NaNO₃, 51 mM Na-gluconate, and 10 mM MES-Tris (pH 5.6) (Sun et al., 2016). Whole-oocyte currents were recorded using a step voltage protocol. The membrane voltage with 7 s duration for each voltage was stepped from +40 to -140 mV with a 20 mV decrement, and the holding potential was 0 mV. The channel currents were recorded using a 900 A two-electrode voltage-clamp amplifier (Axon, USA) connected to a personal computer through a 1440 A interface (Axon). The glass pipettes were prepared using a glass capillary puller (model PC-10, Narishige, Japan) and were filled with 3 M KCl as pipette solution. The software pClamp10.0 (Axon) was used for data acquisition and analysis.

2.5. Extraction and determination of nitrate contents

Wild-type, *slah2-1* mutant, and transgenic *Arabidopsis* plants were grown in hydroponic solution for 4 weeks (Li et al., 2010). For the determination of nitrate concentration in roots and shoots, nitrate was extracted from the plant tissues in boiling water and determined by high-performance liquid chromatography (HPLC) (Agilent 1200 series) using a PARTISIL 10 strong anion-exchange column (Whatman) as described (Chiu et al., 2004; Meng et al., 2016). For the determination of nitrate in the xylem sap, all rosette leaves of plants were removed, the inflorescence stems were cut using a sharp razor, and xylem sap was collected for 6 h as described (Sunarpi et al., 2005). Nitrate contents were determined using the HPLC method.

2.6. Accession numbers

Gene sequence information in this article can be found in the GenBank database using accession numbers AT4G27970 (*AtSLAH2*), AT3G10660 (*AtCPK2*), AT4G23650 (*AtCPK3*), AT4G09570 (*AtCPK4*), AT5G12480 (*AtCPK7*), AT1G18890 (*AtCPK10*), AT1G35670 (*AtCPK11*), and AT3G57530 (*AtCPK32*).

The *slah2-1* mutant (CS907465) was obtained from the ABRC with T-DNA inserted in the first intron.

3. Results and discussion

3.1. Activation of SLAH2 by the constitutively active version of CPK2

To investigate the effect of root-expressed CPKs on SLAH2 activity, we selected 1–3 CPK isoforms from each CPK subgroup as representatives and coexpressed the CPKs with SLAH2 in *Xenopus* oocytes. From subgroup 1, CPK2,

CPK4, and CPK11 were selected. These CPKs have been reported to display enhanced activity with an increase of Ca²⁺ concentration (Boudsocq et al., 2012). We first tested the activation of SLAH2 by CPK2ΔEF. Significantly large S-type anion currents were recorded upon coexpression of CPK2ΔEF with SLAH2 in oocytes (t-test, P < 0.01) and those anion currents were strongly inhibited by S-type anion channel inhibitor DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) (Figure S1). We also recorded SLAH2 currents in nitrate-, chloride-, sulfate-, and malate-based bath solutions. Significantly larger currents were recorded in 50 mM nitrate-based bath solution compared to other anion-based bath solutions (t-test, P < 0.01) (Figure S2), demonstrating that SLAH2 is nitrate-selective. This finding is consistent with a previous report (Maierhofer et al., 2014). Therefore, CPK2ΔEF was used as a positive control in further voltage clamping experiments.

When SLAH2 was coexpressed with the CPK2, CPK4, or CPK11 at a [cRNA] ratio of 1:1, no or only very weak SLAH2-derived currents were observed (Figures 1A and 1B). We then constructed constitutively active versions of these kinases. According to previous reports, the deletion of the EF hands of CPKs renders the kinases Ca²⁺-insensitive and thus leads to a constitutively active form of the CPKs (Geiger et al., 2010, 2011; Scherzer et al., 2012; Maierhofer et al., 2014). We then generated the truncated versions of the CPK2, CPK4, and CPK11 by deleting their EF hand domains and named these truncated CPKs CPK2ΔEF, CPK4ΔEF, and CPK11ΔEF, respectively. Further voltage clamping results showed that no S-type anion channel current was observed in oocytes coexpressing SLAH2 and either CPK4ΔEF or CPK11ΔEF at a [cRNA] ratio of 1:1 (Figures 1A and 1B). Interestingly, instantaneous significantly larger macroscopic currents were recorded in the oocytes coexpressing SLAH2 and CPK2ΔEF (t-test, P < 0.01) (Figures 1A and 1B) while the coexpression of other genes caused neither any mistargeting of an ion channel protein nor repression of the expression of the channel gene in *Xenopus* oocytes (Geiger et al., 2009; Lee et al., 2009; Zhang et al., 2015; Zhang et al., 2016). Our data indicate that the changes of SLAH2's channel activity could have resulted from the changes of the channel activity in *Xenopus* oocytes, as previously reported (Geiger et al., 2009; Brandt et al., 2012; Lefoulon et al., 2014). These results demonstrate that only the constitutively active form of CPK2 activates SLAH2, whereas native as well as constitutively active versions of CPK4 and CPK11 were not able to activate SLAH2 (Figure 1). These data indicate that CPKs from subgroup 1 show distinct properties from each other and may target distinct substrates. As CPK4 and CPK11 exhibit 95% homology in amino acid residue sequences, it should not be surprising to observe the similarities in their substrate specificity.

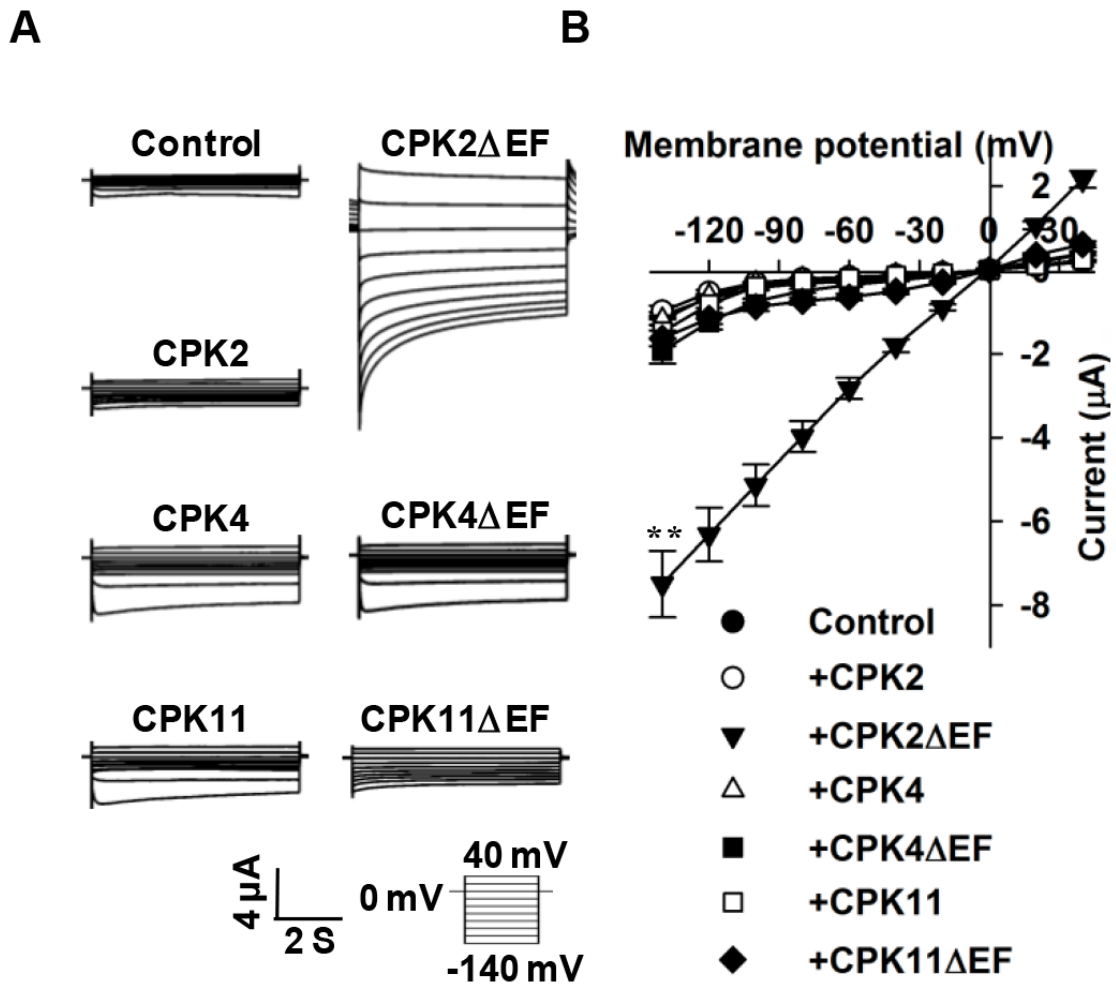


Figure 1. Constitutively active CPK2 activates SLAH2, whereas CPK4 and CPK11 show no obvious effect on the activation of SLAH2. (A) Typical whole-oocyte recordings of S-type anion currents in oocytes injected with water as a control (top left) or SLAH2 cRNA plus the cRNA of a kinase as indicated. (B) Average current-voltage curves of steady-state whole-oocyte currents. The numbers of the oocytes tested are 7 for the control, 5 for SLAH2+CPK2, 11 for SLAH2+CPK2ΔEF, 6 for SLAH2+CPK4, 7 for SLAH2+CPK4ΔEF, 8 for SLAH2+CPK11, and 16 for SLAH2+CPK11ΔEF. Error bars depict means \pm SEM. ** denotes a statistical difference ($P < 0.01$) according to Student's t-test.

3.2. Activation of SLAH2 by native as well as constitutively active forms of CPK3

Subgroup 2 of the CPK family is composed of CPK3, CPK9, and CPK19. These protein kinases are shown to be Ca^{2+} -dependent with lower Ca^{2+} sensitivities relative to subgroup 1 (Boudsocq et al., 2012). We selected CPK3 as a representative from subgroup 2 and tested its effect on the activity of SLAH2. Large S-type anion channel currents were observed in oocytes coexpressing CPK3 and SLAH2 compared with the negative control (Figure 2). To gain further insights, a truncated version of CPK3, i.e. CPK3ΔEF, was coexpressed with SLAH2 at a [cRNA] ratio of 1:1. No significant difference in current amplitudes was observed when the voltage was more negative than -60 mV compared to native CPK3-activated

SLAH2 currents (t-test, $P = 0.826$) (Figures 2A and 2B). These data show that both native as well as truncated CPK3 activates SLAH2 in *Xenopus* oocytes, suggesting a possible Ca^{2+} -independent activity of CPK3. This apparent Ca^{2+} independence of CPK3 is not in agreement with a previous report in which subgroup 2 CPKs, including CPK3, exhibited modest Ca^{2+} sensitivity (Boudsocq et al., 2012). Our finding also differs from a previous report that showed that native CPK3 failed to activate SLAC1 at low Ca^{2+} levels in *Xenopus* oocytes (Scherzer et al., 2012), suggesting that CPK3 could activate SLAH2 and SLAC1 with different Ca^{2+} sensitivities. It has been reported that a CPK might manifest different sensitivities to Ca^{2+} for different substrates (Boudsocq et al., 2012), supporting this conclusion.

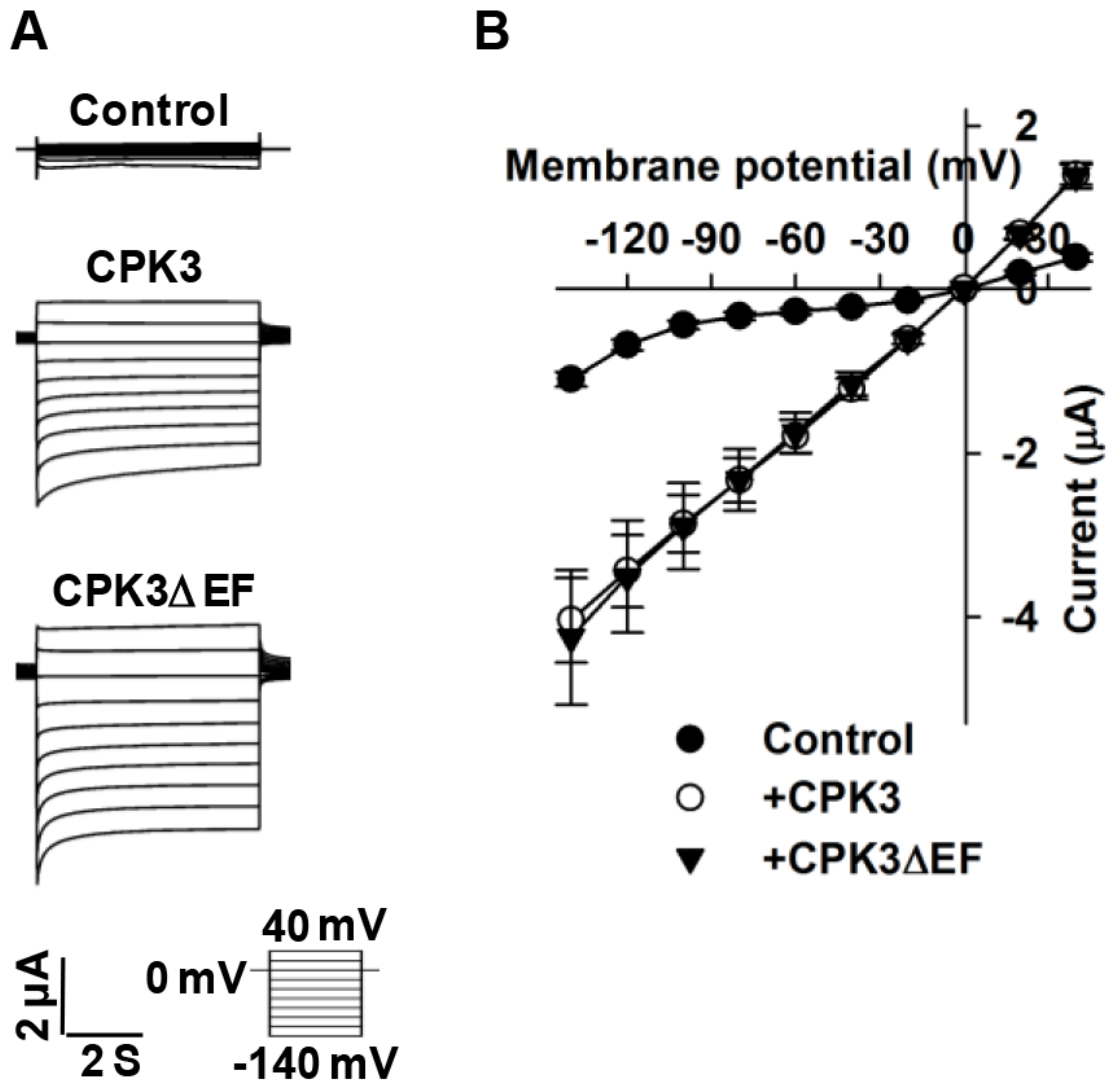


Figure 2. Native as well as truncated CPK3 from subgroup 2 activates SLAH2. (A) Typical whole-oocyte recordings of S-type anion currents in oocytes injected with water as a control or SLAH2 cRNA plus the cRNA of a kinase as indicated. (B) Average current-voltage curves of steady-state whole-oocyte currents. The numbers of oocytes tested are 7 for the control, 8 for SLAH2+CPK3, and 5 for SLAH2+CPK3 Δ EF. Error bars depict means \pm SEM.

3.3. Activation of SLAH2 by native and constitutively active CPKs from subgroup 3

There are 6 CPK members in subgroup 3, namely CPK7, 8, 10, 13, 30, and 32. It has been reported that CPK7, 13, and 30 are Ca^{2+} -independent, whereas CPK8, 10, and 32 are weakly Ca^{2+} -dependent (Boudsocq et al., 2012). We selected CPK7, 10, and 32 as representatives of both subtypes to test their activating effects on SLAH2. Significantly larger SLAH2 anion channel currents were readily elicited by CPK7, CPK10, and CPK32 at the resting cytosolic Ca^{2+} level in the oocytes compared with the negative control (t-test, $P < 0.01$) (Figures 3A and 3B). Next, truncated variants of these CPKs were coexpressed with SLAH2 in *Xenopus* oocytes and anion channel currents

were recorded. We found that SLAH2 was activated by CPK7 Δ EF, CPK10 Δ EF, or CPK32 Δ EF slightly stronger than the native form of these CPKs (Figures 3A and 3B). However, this difference could be possibly associated with the conformational changes of the CPKs due to the deletion of their EF hands. Taken together, these findings demonstrate that both native and truncated versions of the three selected CPKs from subgroup 3 can activate SLAH2 in *Xenopus* oocytes. Although these findings provide us a clue about the apparent calcium-independent activity of the tested CPKs for SLAH2 activation in *Xenopus* oocytes, further research is still needed to validate these findings in vivo. These data are consistent with previous reports showing that CPKs from subgroup 3 activate substrates

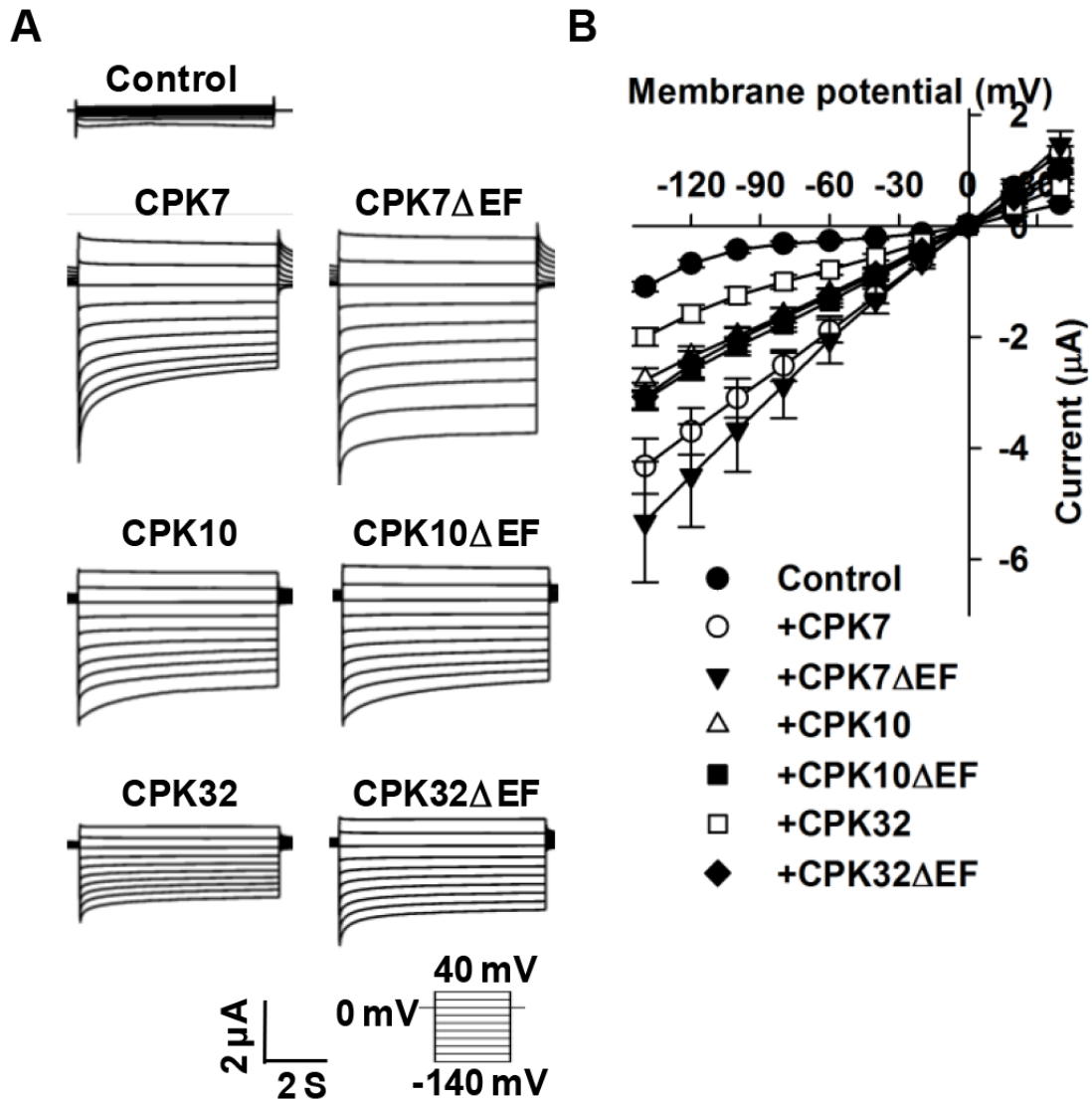


Figure 3. Both native and truncated versions of CPK7, CPK10, and CPK32 from subgroup 3 activate SLAH2. (A) Typical whole-oocyte recordings of S-type anion currents in oocytes injected with water as a control or *SLAH2* cRNA plus the cRNA of a kinase as indicated. (B) Average current-voltage curves of steady-state whole-oocyte currents. The numbers of oocytes tested are 7 for the control, 11 for *SLAH2*+CPK7, 10 for *SLAH2*+CPK7 Δ EF, 11 for *SLAH2*+CPK10, 6 for *SLAH2*+CPK10 Δ EF, 14 for *SLAH2*+CPK32, and 8 for *SLAH2*+CPK32 Δ EF. Error bars depict means \pm SEM.

with low Ca^{2+} sensitivities or even exhibit insensitivity to Ca^{2+} (Boudsocq et al., 2012). The CPKs from subgroup 3 have been reported to be different from the rest of the subgroups with respect to their Ca^{2+} dependence. Although all of the subgroup 3 CPKs exhibited normal Ca^{2+} binding at EF hands, their activity was only slightly Ca^{2+} -sensitive (Boudsocq et al., 2012). It has also been speculated that Ca^{2+} binding at the EF hands of subgroup 3 CPKs could be required for other regulatory processes like lipid binding and protein-protein interaction (Klimecka and Muszynska, 2007; Zou et al., 2010).

3.4. Nitrate contents of *slah2-1* mutant and SLAH2 overexpression lines are not obviously different from the wild type

The release of ions into the xylem vessels takes place through root stele cells in plants. Xylem-slowly activating anion conductance (X-SLAC) has been reported in the plasma membranes of stele cells from barley roots (Kohler and Raschke, 2000). SLAH2 is expressed in the stele cells of *Arabidopsis* roots (Maierhofer et al., 2014). Owing to its strict nitrate selectivity, a role of SLAH2 in nitrate transport in roots has been hypothesized (Maierhofer et

al., 2014). It is speculated that a channel like SLAH2 with strict nitrate selectivity could be advantageous for plants, especially in salt-stress conditions where selective nitrate transport is required (Maierhofer et al., 2014). We were keen to investigate whether SLAH2 plays any role in nitrate transportation in *Arabidopsis*. We acquired a T-DNA insertion mutant *slah2-1* (Figure 4A). We generated two

independent genetic complementation lines, COM4 and COM5, by expressing *SLAH2* driven by a 35S promoter in the *slah2-1* mutant background. In addition, two overexpression lines, OE1 and OE7, were also generated by expressing *SLAH2* under a 35S promoter in the wild-type background. The expression levels of *SLAH2* in *slah2-1* mutants, the transgenic lines, and wild-type plants

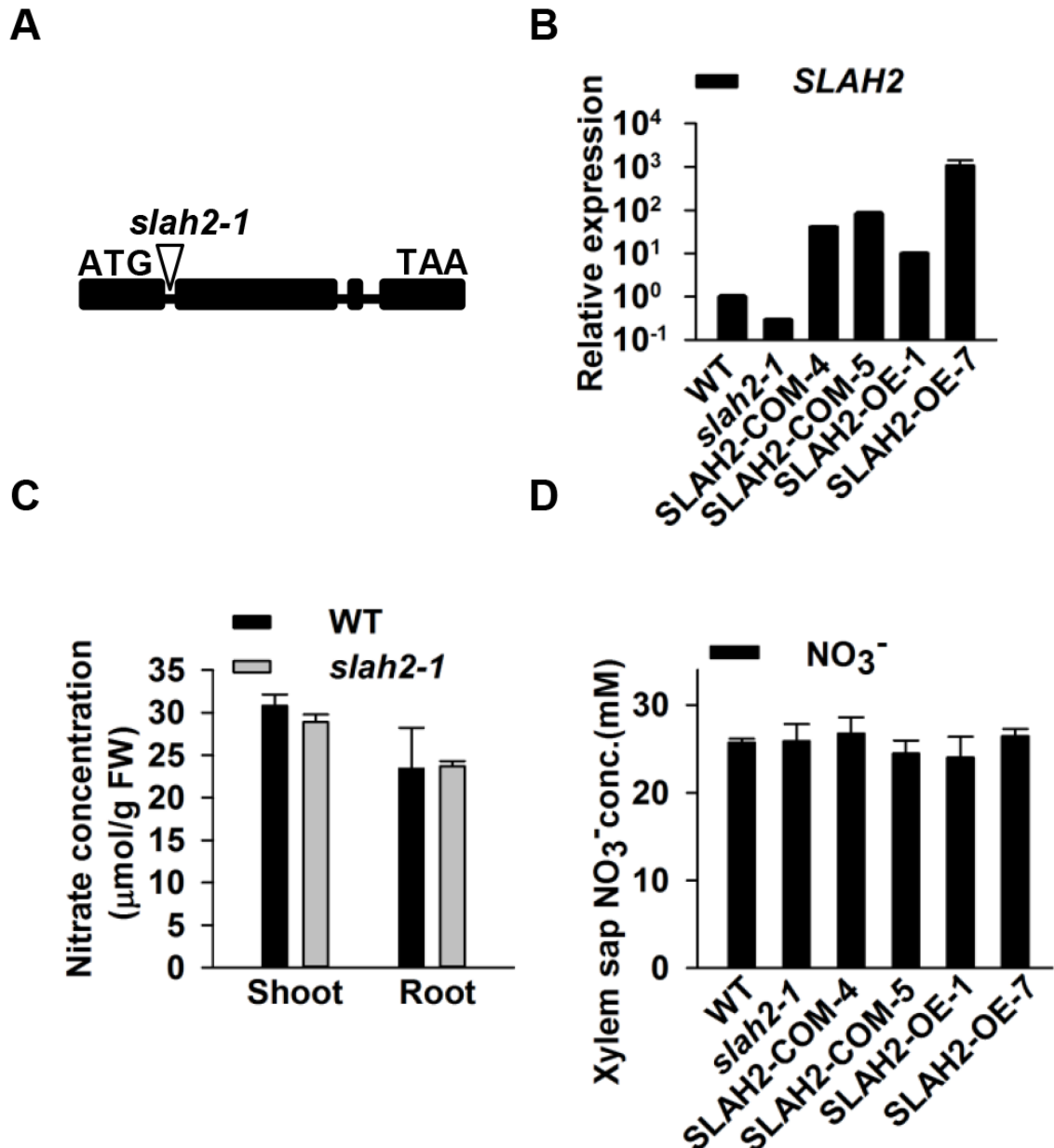


Figure 4. Determination of nitrate contents in wild-type, *slah2-1*, and transgenic lines. (A) Scheme of the exon-intron gene structure of *AtSLAH2* (AT4G27970) showing the T-DNA insertion site in the first intron in the *slah2-1* mutant. (B) qRT-PCR data showing the *SLAH2* expression level in the *slah2-1* mutant, overexpression lines, and complementation lines. (C) The nitrate content in the shoots and roots of the *slah2-1* mutant and the wild type (23-day-old plants). (D) The nitrate content in xylem sap of *slah2-1* mutant, genetic complementation lines, and *SLAH2* overexpression lines (23-day-old plants). Error bars depict means \pm SEM. The experiment was repeated three times.

were tested using the qRT-PCR technique (Figure 4B). We then determined the nitrate contents in the roots and shoots of *slah2-1* and wild-type plants and found that the nitrate contents in shoots were slightly higher than in roots (Figure 4C), which is in agreement with a previous report (Li et al., 2010). However, no significant difference of nitrate contents was observed between *slah2-1* and wild-type plants (Figure 4C). These data indicate that the mutation of SLAH2 might not be significant to affect the overall nitrate content of the *Arabidopsis* shoots and roots. To explore the potential role of SLAH2 in nitrate loading into xylem sap, we quantified nitrate contents in the xylem sap of *slah2-1*, transgenic lines, and the wild type. The nitrate contents of the *slah2-1* mutant, the complementation lines, and overexpression lines were not significantly different compared to the wild type (t-test, $P = 0.959, 0.699, 0.614, 0.592, \text{ and } 0.716$ for *slah2-1* mutant, COM4, COM5, OE1, and OE7, respectively) (Figure 4D). We also attempted to find the phenotype of the *slah2-1* mutant grown in salt-stress conditions. Seed germination as well as mature plants' growth was not obviously different from control plants under salt-stress conditions (Figure S3). These findings suggest that SLAH2 could play a less significant role, if any, in nitrate transport in the conditions tested. NRT1.5, a low-affinity

nitrate transporter, has been reported to be involved in nitrate loading to xylem vessels, but nitrate transport from roots to shoots showed no difference between the *nrt1.5* mutant and the wild type (Lin et al., 2008). In such a condition, other nitrate transporters could be involved in nitrate transport (Wang et al., 2012). NRT1.8 and NRT1.9 are reported to be involved in nitrate translocation from shoots back to roots (Li et al., 2010; Wang and Tsay, 2011). This could be to ensure that excessive nitrate is not overaccumulated in aerial parts, especially in nitrate-rich environments. Therefore, root-to-shoot nitrate transport is a key step in determining nitrate distribution. For this reason, nitrate translocation could be a finely tuned process with the involvement of multiple nitrate transporters and/or channels. Further research will be needed to clarify the role of SLAH2 in nitrate transport.

In conclusion, we have reported a few root-expressed CPKs from 3 subfamilies that are capable to activate SLAH2 with diverse Ca^{2+} sensitivity. Moreover, we also attempted to explore the role of SLAH2 in nitrate transport in vivo. However, knocking out as well as overexpression of SLAH2 did not significantly alter the nitrate contents of xylem sap of *Arabidopsis* plants. Further research will be required for a clear understanding of the biological functions of SLAH2 in *Arabidopsis*.

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Table S1. List of primers.

Vectors	Primers	Primer sequence (5' to 3')	Purpose
pGEMHE	<i>SLAH2</i> -pGE-F	CCCCCGGGATGAATAATCCAAGATCGGTT	Cloning of <i>SLAH2</i>
	<i>SLAH2</i> -pGE-R	CCCAAGCTTTTAATTTGAATTCTGAACCGT	
pGEMHE	<i>CPK2</i> -pGE-F	TTCCCGGG ATGGGTAATGCTTGCGT	Cloning of <i>CPK2</i>
	<i>CPK2</i> -pGE-R	AA GGATCC CTAATGTTTCAGAGAA	
pGEMHE	<i>CPK2</i> -pGE-F	TTCCCGGG ATGGGTAATGCTTGCGT	Cloning of <i>CPK2-ΔEF</i>
	<i>CPK2</i> -pk-R	TTGGATCCCTATACCCATGGATGACATAA	
pGEMHE	<i>CPK3</i> -F	TTCCCGGGATGGGCCACAGACACAGCAA	Cloning of <i>CPK3</i>
	<i>CPK3</i> -pGE-R	TTGGATCCTCACATTCTGCGTCGGTTTG	
pGEMHE	<i>CPK3</i> -F	TTCCCGGGATGGGCCACAGACACAGCAA	Cloning of <i>CPK3-ΔEF</i>
	<i>CPK3</i> -pk-R	TTGGATCCTCACTCCCCATCTTCTCTAA	
pGEMHE	<i>CPK4</i> -F	AACCCGGGATGGAGAAACCAAACCCTAGA	Cloning of <i>CPK4</i>
	<i>CPK4</i> -pGE-R	AATCTAGATTACTTTGGTGAATCATCAGATTT	
pGEMHE	<i>CPK4</i> -F	AACCCGGGATGGAGAAACCAAACCCTAGA	Cloning of <i>CPK4-ΔEF</i>
	<i>CPK4</i> -pk-R	AATCTAGATTAAGCATGTTTCATCAACAA	
pGEMHE	<i>CPK7</i> -F	AACCCGGGATGGGGAATTGTTGTGGC	Cloning of <i>CPK7</i>
	<i>CPK7</i> -pGE-R	TTGGATCCTTAGGTCTCGCCTTCTAATT	
pGEMHE	<i>CPK7</i> -F	AACCCGGGATGGGGAATTGTTGTGGC	Cloning of <i>CPK7-ΔEF</i>
	<i>CPK7</i> -pk-R	TTGGATCCTTATATCCAAGTATGTTTCGAG	
pGEMHE	<i>CPK10</i> -F	TTCCCGGGATGGGTAACGTGAACGCCTGT	Cloning of <i>CPK10</i>
	<i>CPK10</i> -pGE-R	TTTCTAGATTAACAGGAACAGTTTGTCCAGT	
pGEMHE	<i>CPK10</i> -F	TTCCCGGGATGGGTAACGTGAACGCCTGT	Cloning of <i>CPK10-ΔEF</i>
	<i>CPK10</i> -pk-R	TTTCTAGATTAATAGACAAGTGCTCCGCA	
pGEMHE	<i>CPK11</i> -F	TTCCCGGGATGGAGACGAAGCCAAACCCT	Cloning of <i>CPK11</i>
	<i>CPK11</i> -pGE-R	TTGGATCCTCAGTCATCAGATTTTTCACCATC	
pGEMHE	<i>CPK11</i> -F	TTCCCGGGATGGAGACGAAGCCAAACCCT	Cloning of <i>CPK11-ΔEF</i>
	<i>CPK11</i> -pk-R	TTGGATCCTCAAATCCATGGGTGACACAAGG	
pGEMHE	<i>CPK32</i> -F	AACCCGGGATGGGTAATGTTGCGGA	Cloning of <i>CPK32</i>
	<i>CPK32</i> -pGE-R	AAGGATCCTCATCTTGATCACCATT	
pGEMHE	<i>CPK32</i> -F	AACCCGGGATGGGTAATGTTGCGGA	Cloning of <i>CPK32-ΔEF</i>
	<i>CPK32</i> -pk-R	TTGTCGACTCATAACCACGGGTGATCTAG	
pCAMBIA1300S	<i>SLAH2</i> -F	CCCAAGCTTTAATGAATAATCCAAGATCGG	Cloning of <i>SLAH2</i>
	<i>SLAH2</i> -R	CCCGTCGACTTAATTTGAATTCTGAACCGT	
	<i>SLAH2</i> -F	CCAGTGCACGATCA AA	qRT-PCR
	<i>SLAH2</i> -R	CCGTGGAACCTATCTACA	
	<i>18S</i> -F	CGGCTACCACATCCAAGGAA	qRT-PCR
	<i>18S</i> -R	TGTCACTACCTCCCCGTGTCA	

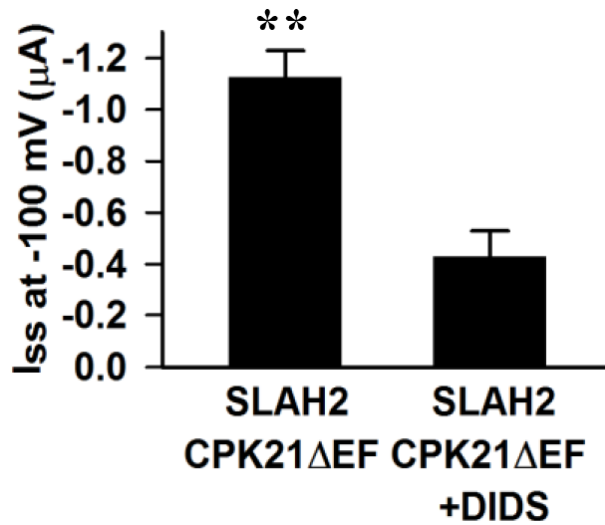


Figure S1. CPK21DEF-activated SLAH2-mediated S-type anion channel currents at -100 mV are significantly reduced by the application of 0.1 mM DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid). The numbers of the oocytes tested are 3 for each condition. Error bars depict means \pm SEM. ** denotes statistical difference (t-test, $P < 0.01$).

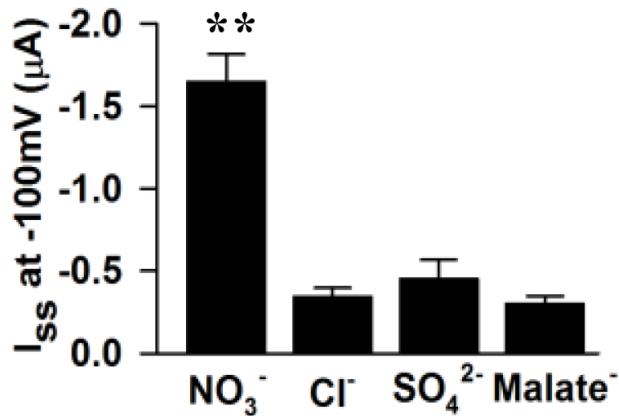
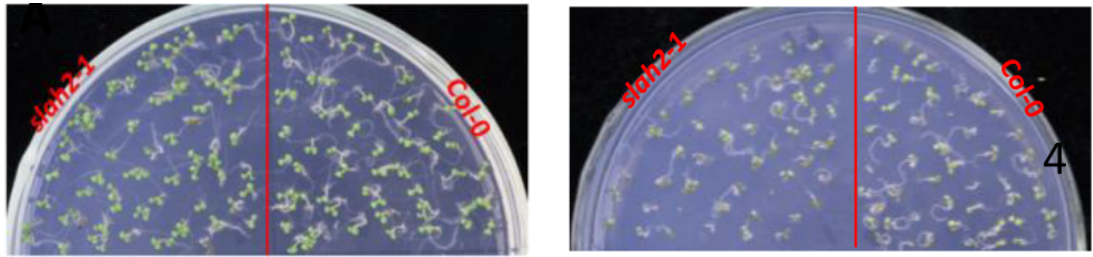


Figure S2. SLAH2 exhibits larger permeability to nitrate relative to chloride, sulfate, and malate. SLAH2-mediated S-type anion channel currents were activated by CPK21DEF in NO_3^- , Cl^- , SO_4^{2-} , and malate $^-$ -based bath solutions at a concentration of 50 mM, respectively. The numbers of the oocytes tested are 8 for NO_3^- , 9 for Cl^- , 7 for SO_4^{2-} , and 6 for malate $^-$. Error bars depict means \pm SEM. ** denotes statistical difference (t-test, $P < 0.01$).



0 mM NaCl

125 mM NaCl



0 mM NaCl

125 mM NaCl

200 mM NaCl

Figure S3. *slah2-1* and Col-0 grown in salt-stress condition. (A) Seed germination assay on MS media supplemented with 0 or 125 mM NaCl for 1 week. (B) The growth of *slah2-1* mutant and wild-type plants in hydroponic solution supplemented with NaCl in a concentration as indicated. In each pot, Col-0 is on the left side and *slah2-1* is on the right side.