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SLAH1, a homologue of the slow type anion channel SLAC1, modulates shoot Cl\(^{-}\) accumulation and salt tolerance in *Arabidopsis thaliana*

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Abstract

Salinity tolerance is correlated with shoot chloride (Cl\(^{-}\)) exclusion in multiple crops, but the molecular mechanisms of long-distance Cl\(^{-}\) transport are poorly defined. Here, we characterize the *in planta* role of AtSLAH1 (a homologue of the slow type anion channel-associated 1 (SLAC1)). This protein, localized to the plasma membrane of root stelar cells, has its expression reduced by salt or ABA, which are key predictions for a protein involved with loading Cl\(^{-}\) into the root xylem. Artificial microRNA knockdown mutants of *AtSLAH1* had significantly reduced shoot Cl\(^{-}\) accumulation when grown under low Cl\(^{-}\), whereas shoot Cl\(^{-}\) increased and the shoot nitrate/chloride ratio decreased following *AtSLAH1* constitutive or stelar-specific overexpression when grown in high Cl\(^{-}\). In both sets of overexpression lines a significant reduction in shoot biomass over the null segregants was observed under high Cl\(^{-}\) supply, but not low Cl\(^{-}\) supply. Further *in planta* data showed *AtSLAH3* overexpression increased the shoot nitrate/chloride ratio, consistent with *AtSLAH3* favouring nitrate transport. Heterologous expression of *AtSLAH1* in *Xenopus laevis* oocytes led to no detectible transport, suggesting the need for post-translational modifications for *AtSLAH1* to be active. Our *in planta* data are consistent with *AtSLAH1* having a role in controlling root-to-shoot Cl\(^{-}\) transport.

Key words: ABA, Arabidopsis, AtSLAH1, AtSLAH3, chloride, Cl\(^{-}\) xylem loading, long-distance transport, nutrition, salinity, slow-type anion channel-associated homologue 1, slow-type anion channel-associated homologue 3.

Introduction

Chloride (Cl\(^{-}\)) is classified as a micronutrient, but it is often present in plant tissues at concentrations typical of a macronutrient (i.e. 2–20 rather than 0.1–200 \(\mu\)g g\(^{-1}\) DW) (Marschner, 1995; Xu *et al.*, 2000; Broadley *et al.*, 2012; Franco-Navarro *et al.*, 2015). Cl\(^{-}\) has vital roles in regulating numerous physiological processes including turgor, enzyme activity, photosynthesis and membrane potential (Rognes, 1980; White and Broadley, 2001; Teakle and Tyerman, 2010). Although the pathways for Cl\(^{-}\) entry and movement within the plant have been characterized biochemically, their molecular determinants are poorly defined (Teakle and Tyerman, 2010; Henderson *et al.*, 2014).

High concentrations of sodium chloride (NaCl) in soils reduces crop yield (Rengasamy, 2010, Roy *et al.*, 2014), which
can impose significant economic costs to farmers (Munns and Gilliham, 2015). Na\(^+\) transport and its impact on plant growth have been relatively well documented at both a physiological and a molecular level in a variety of plant species (Blumwald et al., 2000; Zhu, 2003; Horie and Schroeder, 2004; Davenport et al., 2005; Garthwaite et al., 2005; Chinnusamy et al., 2006; Apse and Blumwald, 2007; Horie et al., 2008; Müller et al., 2014; Roy et al., 2014; Maathuis et al., 2014; Flowers et al., 2015). However, in other economically important crop plants like soybean, grapevine, citrus and lotus, leaf Cl\(^-\) accumulation (not Na\(^+\)) is correlated with decreased plant growth and photosynthesis when plants are under salt stress (Storey and Walker, 1999; Walker et al., 2002; Tregeagle et al., 2006; Teakle et al., 2007; Teakle and Tyerman, 2010; Gong et al., 2011). So, although Cl\(^-\) is a micronutrient it also can accumulate to concentrations that inhibit plant growth when plants encounter salinity. The cause of both Na\(^+\)- and Cl\(^-\)-induced reductions in photosynthesis and growth, and the cause of salt-induced cell death are yet to be definitively determined and are a priority area for research (Munns and Gilliham, 2015). Some studies have investigated the relative impact of Na\(^+\) and Cl\(^-\) on barley and wheat (e.g. Tavakkoli et al., 2011; Genc et al., 2015). Growth and photosynthesis of several cultivars of barley appeared to be more sensitive to the addition of Cl\(^-\) than of Na\(^+\) (Tavakkoli et al., 2011). These findings highlight the importance of investigating the regulation of both Na\(^+\) and Cl\(^-\) transport for improving plant salt tolerance, even in species that are classically considered to be more Na\(^+\)-sensitive than Cl\(^-\)-sensitive under saline conditions.

Identification of genes that underpin root-to-shoot Cl\(^-\) transport, and the related signalling pathways, should provide information that could be used to reduce Cl\(^-\) sensitivity in commercial crops. A key pathway in controlling Cl\(^-\) accumulation in the shoot is its loading from xylem parenchyma cells into the transpiration stream (Teakle and Tyerman, 2010). Recently, a nitrate (NO\(_3\)\(^-\)) transporter 1/peptide transporter family member (NPF2.4) was identified as the first protein to be directly involved in loading Cl\(^-\) into the root xylem (Li et al., 2016). However, Cl\(^-\) accumulation in the shoot is predicted to be a multigenic trait in a number of plant groups and species including soybean, maize, grapevine, citrus and legumes (Abel, 1969; Storey and Walker, 1999; Moya et al., 2003; Sibole et al., 2003; Gilliham and Tester, 2005; Gong et al., 2011; Henderson et al., 2014; Fort et al., 2015). Knockouts of Atnptf2.4 had a 20% reduction in shoot Cl\(^-\) (Li et al., 2016), providing further evidence of the multigenic nature of shoot Cl\(^-\) accumulation. Therefore, other anion transport proteins are likely to be involved in root-to-shoot Cl\(^-\) transport (e.g. Henderson et al., 2014), but these remain to be identified and functionally characterized at a molecular level.

Three anion conductances have been identified using electrophysiology in barley root xylem parenchyma protoplasts, namely an inwardly rectifying anion channel (X-IRAC), a quickly activating anion conductance (X-QUAC) and a slowly activating anion conductance (X-SLAC) (Köhler and Raschke, 2000). Similar results were found in maize root stelar cells (Gilliham and Tester, 2005) and Arabidopsis root pericycle cells (Kiegle et al., 2000). X-QUAC is the most prevalent conductance observed in xylem parenchyma cells and is likely to load the majority of Cl\(^-\) (and NO\(_3\)\(^-\)) ions into the xylem under non-saline conditions (Köhler et al., 2002; Gilliham and Tester, 2005) as the estimated flux through this conductance could easily account for the Cl\(^-\) release from the xylem vessels measured using a \(^{36}\)Cl tracer (Pitman, 1982; Köhler and Raschke, 2000).

The hormone abscisic acid (ABA) regulates solute transport from root to shoot (Cram and Pitman, 1972). Excised barley roots treated with ABA for 2h accumulated significantly more Cl\(^-\) than untreated roots (Cram and Pitman, 1972). Cl\(^-\) efflux to the xylem was also reduced following the ABA treatment, but Cl\(^-\) influx into the root was unaffected (Cram and Pitman, 1972). These results indicate that ABA down-regulates xylem loading of Cl\(^-\) in roots but not root Cl\(^-\) influx. Furthermore, the anion conductances in maize, barley and Arabidopsis stele, as well as the potassium conductance through the stelar K\(^+\) outwardly rectifying channel (SKOR), are also down-regulated by ABA (Cram and Pitman, 1972; Gilliham and Tester, 2005). In Arabidopsis, AtSKOR was transcriptionally down-regulated by ABA (Gaymard et al., 1998). Therefore, it may be possible to identify candidate genes for Cl\(^-\) loading into the root xylem by characterizing those genes encoding putative anion transporters that are expressed in the stele and are down-regulated (either transcriptionally or post-translationally) by ABA.

Early electrophysiological studies on stomatal guard cells revealed the slowly activated anion conductance (SLAC) (Linder and Raschke, 1992). More recently, the gene encoding the protein responsible for this conductance, SLAC1, was identified (Negi et al., 2008; Vahisalu et al., 2008). SLAC1 is a plasma membrane (PM) localized protein, highly permeable to malate and chloride (Negi et al., 2008; Chen et al., 2010), and slac1 mutants have increased Cl\(^-\) in guard cells (Negi et al., 2008; Vahisalu et al., 2008). Four homologues of SLAC1, the slow-type anion channel-associated homologues 1 to 4 (SLAH1 to 4), have been identified that also localize to the PM and are predicted to be involved in anion transport (Negi et al., 2008; Vahisalu et al., 2008). Expression of SLAH1 or SLAH3 in slac1 knockout mutants could complement both the defective closure response of slac1 stomata to high CO\(_2\) and its ionic profile when constitutively overexpressed (Negi et al., 2008). SLAH3 is expressed in guard cells and roots, and is preferentially selective for NO\(_3\) over Cl\(^-\); it has a role in NO\(_3\)- alleviation of ammonium toxicity (Geiger et al., 2009, 2011; Demir et al., 2013; Zheng et al., 2015). SLAH2 is also expressed in roots (Maierhofer et al., 2014), and the protein is predominantly permeable to NO\(_3\) (with a NO\(_3\)/Cl\(^-\) permeability ratio of 82) (Maierhofer et al., 2014). Both SLAH2 and SLAH3 have been predicted to have roles in loading NO\(_3\) into the root xylem (Maierhofer et al., 2014; Zheng et al., 2015). SLAH1 is also expressed in the root; however, the role of AtSLAH1 is currently unknown. As SLAH1 is expressed in the root stele and can complement the stomatal phenotype of the Atslac1 mutant when ectopically expressed (Negi et al., 2008; Vahisalu et al., 2008; Zheng et al., 2015), we examined whether AtSLAH1 has a role in loading Cl\(^-\) into the root xylem.
Materials and methods

Plant materials and growth conditions

All chemicals were obtained from Sigma-Aldrich unless stated. Arabidopsis thaliana ecotype (Col-0) seeds were purchased from the European Arabidopsis Stock Centre (Nottingham, UK). Plants were grown within temperature controlled growth rooms. Arabidopsis plants grown in soil were kept in long day conditions (16 h day/8 h night), while those in hydroponics were kept in short day conditions (10 h day/14 h night). In both long day and short day conditions, the temperature was maintained at 21–23 °C, the humidity was maintained between 60–75%, and the irradiance during the light period was 150 μmol m⁻² s⁻¹. Plants were grown in hydroponics following protocols described in Conn et al. (2013) and in soil following methods described in Møller et al. (2009).

Generation of AtSLAH1 artificial microRNA lines

The AtSLAH1 T-DNA knockout mutant (FLAG_329G06) was ordered through the Versailles Arabidopsis Stock Centre; however, the expression of AtSLAH1 (At1G62280) was detectable in all homozygous mutant lines (Supplementary Fig. S1 at JXB online). To elucidate the function of AtSLAH1 in planta, artificial microRNAs (amiRNAs) were designed to knockdown AtSLAH1 expression. To produce AtSLAH1 knocked down mutants, specific amiRNAs were designed against the AtSLAH1 mRNA sequence using Micro RNA Designer (http://www3.weigelworld.org/cgi-bin/webapp.cgi) following the protocol of Schwab et al. (2006). Two 21 bp target sequences (TAAAGCGCTATTTGGTTCCGT and TTATGTCTAGTGTCGAGACTG) were identified from the AtSLAH1 coding sequence and two independent amiRNA constructs were generated with a set of primers (Supplementary Table S1) to incorporate the 21bp amiRNA sequence into the MIR319a vector (Schwab et al., 2006). Both full-length SLAH1-amiRNA products were cloned using high-fidelity Phusion® polymerase (New England Biolabs, USA) into a Gateway® enabled pCR8 entry vector (Invitrogen, CA, USA) and transferred into the pMDC32 expression vector (Curtis and Grossniklaus, 2003) through an LR reaction (Invitrogen). The constructs were transformed into Arabidopsis using Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998). Hygromycin B (20 μg ml⁻¹) was used to select the transformants following the protocol described in Harrison et al. (2006).

Generation of cell type-specific overexpression lines

An Arabidopsis enhancer trap line (E2568; Møller et al., 2009) was used to generate plants with AtSLAH1 root stelar-cell-specific overexpression. AtSLAH1 full length cDNA was cloned using high-fidelity Phusion® polymerase (New England Biolabs, USA) from Arabidopsis root cDNA (following RNA extraction and cDNA synthesis following Henderson et al. (2015)) into a Gateway-enabled pCR8 entry vector (Life Technologies, CA, USA). AtSLAH1 was then transferred into a pTOOL5 destination vector (pMDC132+UAS+SOS) (Plet, 2008) containing the GAL4-inducible promoter UAS, which drives target gene expression specifically in root stelar cells in line E2568. The construct was transformed into Arabidopsis line E2586 using Agrobacterium-mediated transformation (Clough and Bent, 1998). The seeds from transformed plants were harvested and germinated in soil. When the seedling had two to four true leaves, 20 mg l⁻¹ BASTA (Bayer, Germany) was sprayed on the seedlings to select for plants with the transgenic insertion.

Generation of constitutive overexpression lines

Full-length AtSLAH1 and AtSLAH3 coding sequences were amplified (the primers used are listed in Supplementary Table S1) from Arabidopsis root cDNA, using Phusion® polymerase, cloned into the Gateway-enabled pCR8 entry vector (Life Technologies) and transferred into the pMDC32 expression vector (Curtis and Grossniklaus, 2003) through an LR reaction (Life Technologies). The construct was transformed into Arabidopsis (Col-0) using Agrobacterium-mediated transformation (Clough and Bent, 1998). Hygromycin B (20 μg ml⁻¹) was used to select lines containing the transgene insertion following the protocol described in Harrison et al. (2006).

Salinity and ABA treatment

Both salinity and ABA treatment were performed in hydroponics (Conn et al., 2013). For the 7-day salinity treatment, NaCl was added to basal nutrient solution (BNS) (Conn et al., 2013) to make a final concentration of 50, 75, and 100 mM. Additional CaCl₂ was added to each solution to achieve a constant Ca²⁺ activity of 1.3 mM following the addition of high concentrations of monovalent cations, which act to reduce the activity of other cations and induce calcium deficiency (as detailed in Conn et al. (2013)). For ABA treatment, a stock solution of 100 mM (±)-cis-trans-abscisic acid was made in absolute ethanol. When applying 20 μM ABA, this resulted in a final ethanol concentration of 0.01% (v/v) when added into the growth solution.

Expression analysis

Gene expression analysis by qRT-PCR was performed following the method described in Burton et al. (2008). The primers for examining AtSLAH1 expression were 5′-TCTCTATGTCCTCCGTGTCG3′ (forward) and 5′-ATTGCTGTGTGCTGTC3′ (reverse) and for AtSLAH3 were 5′-ATCTCGTGCTGTCGTTGGAACTTTG3′ (forward) and 5′-CTCGTGCTGTCGTTGGAACTTTG3′ (reverse). The selected Arabidopsis housekeeping genes (AtGAPDH (At3G26650), AtActin2 (At3G18780), AtTubulin (At1G50010) and At Cyclophilin (At2G36130)) and data normalization followed the methods described in Iha et al. (2010). For relative gene expression, AtActin2 was used as a control gene, and the relative expression level of target genes was detected using the same primer pairs as listed above using a QuantStudio 12K Flex Real-Time PCR system (Life Technologies).

Phenotyping transgenic plants

For determining the shoot NO₃⁻ concentration, a method that uses salicylic acid to form a chromophore with NO₃⁻ under alkaline conditions (pH > 12) was used (Cataldo et al., 1975). In brief, 3–5 mg of Arabidopsis dried tissue was extracted in 0.5 ml deionized water, with 0.05 ml of the extraction incubated with 0.2 ml of 5% (w/v) salicylic acid–H₂SO₄ for 20 min at room temperature; 0.05 ml of this mixture was transferred into a fresh tube and 0.95 ml of 2 M NaOH was added. A 0.2 ml aliquot was transferred to a well of a transparent 96-well plate and the absorbance at OD₄₅₀nm determined. A serial dilution of known concentrations of potassium nitrate (KNO₃) was used for the standard, which ranged from 0 to 50 mM.

For determining the shoot Cl⁻ concentration, 20–30 mg of freeze-dried Arabidopsis tissue was extracted in 500 μl 1% nitric acid at 80 °C overnight. A chloride analyser (Model 926S, Sherwood Scientific, Cambridge, UK) was used to examine the Cl⁻ concentration by following the manufacturer’s instructions.

Expression and electrophysiological characterization of AtSLAH1 in X. laevis oocytes

The AtSLAH1, AtSnRK2.2, and AtSnRK2.3 coding sequences (primers are listed in Supplementary Table S1) were cloned using high-fidelity Phusion® polymerase (New England Biolabs, USA) from Arabidopsis root cDNA into a Gateway-enabled pCR8 entry vector (Life Technologies) before being transferred into the pGEM-HE (DEST) vector, and cRNA was synthesized using the mMESSAGE mMACHINE® kit (Ambion, Australia) as previously described (Preuss et al., 2011). Healthy stage IV–VI defolicated oocytes were obtained through surgery and enzymatic digestion of ovaries from toads kept
in an *Xenopus* colony at the Waite Campus (University of Adelaide). The cRNA (46 nl/23 ng per oocyte) was injected using a micro injector (Drummond Nanoinject II injector, USA) with a glass microcapillary pipette following the manufacturer’s procedures. The same volume of nuclease-free water was injected into control oocytes. Injected oocytes were incubated at 18 °C for 2 days in an NDF6 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.8 mM CaCl2, pH 7.4 with 1 M Tris) combined with horse serum (50 ml l−1), tetracline (50 μg ml−1) and penicillin (50 μg ml−1). After 2 days, *AtSLAH1* cRNA-injected oocytes were voltage clamped from +40 to –120 mV in 20 mV decrements for 3 s purfusing in the following bath solutions (basal: 2 mM calcium gluconate, 5 mM HEPES and 0.1 mM LaCl3) plus 1 or 20 mM CsNO3/CsCl at pH 7.5. Two-electrode voltage clamping (TEVC) was performed on oocytes as previously described in Roy et al. (2008) using an OC-725C amplifier (Warner Instruments Corp., USA), signals were digitized with a Digidata 1440A (Molecular Devices, USA), and then the data were recorded and analysed using pCLAMP 10.2 (Molecular Devices, USA).

**Results**

*AtSLAH1* expression is down-regulated by both salt and ABA

A qRT-PCR was performed on Arabidopsis root cDNA to determine whether *AtSLAH1* transcript abundance altered following salt or ABA treatment. The expression level of *AtSLAH1* was significantly reduced by 91% after 7 days of 100 mM NaCl treatment, and by 97% after 16 h of 20 μM ABA treatment when compared with the control (Fig. 1A). In contrast, the close homologue *AtSLAH3*, which shares the same cell location in xylem parenchyma and pericycle cells of the root stele and is also PM localized (Negi et al., 2008), was not down-regulated by ABA or salt treatment (Fig. 1B). All these data are consistent with previous observations (Kilian et al., 2007; Brady et al., 2007; Gifford et al., 2008; Supplementary Fig. S2).

*AtSLAH1* amiRNA knockdown lines have low Cl− accumulation in the shoot under low Cl− supply

To investigate whether *AtSLAH1* was involved in root-to-shoot anion transport, different Arabidopsis lines with an increase or decrease in *AtSLAH1* expression were generated. *Atslah1* knockout lines (FLAG_329G06) were ordered from the Versailles Arabidopsis Stock Centre. Homozygous lines were successfully identified (Supplementary Fig. S1); however, RT-PCR performed using *AtSLAH1*-specific primers (Supplementary Table S1) found that the expression of *AtSLAH1* was not abolished in these mutant lines (Supplementary Fig. S1C). Therefore, four independent amiRNA:*AtSLAH1* mutant lines were generated, which were named amiRNA:*AtSLAH1_1* (two inserts), amiRNA:*AtSLAH1_2* (two inserts), amiRNA:*AtSLAH1_3* (three inserts) and amiRNA:*AtSLAH1_4* (two inserts). Under low salt conditions (2 mM NaCl), qRT-PCR showed that the transcript abundance of *AtSLAH1* in the root of all independent amiRNA lines was less than half of that found in the null segregants (*P*≤0.05) (Fig. 2A). In all amiRNA:*AtSLAH1* lines, the shoot Cl− concentration was significantly lower than that of the null segregants under low salt conditions, being reduced by 30–47% (*P*≤0.05) (Fig. 2B). The expression level of *AtSLAH1* was plotted against shoot Cl− concentration for each plant and a highly significant positive relationship was observed with an *R*2 of 0.89. The shoot NO3− concentration under low Cl− supply was also determined and no difference was found between the mutants and the null segregants (Supplementary Fig. S3A), but the reduction in shoot Cl− led to a significantly greater shoot NO3−/Cl− ratio in all mutants when compared with the null segregants (Fig. 2D). No differences were found in shoot biomass in any of the amiRNA:*AtSLAH1* lines under low Cl− conditions (Supplementary Fig. S3B). The shoot Na+ and K+ concentrations were determined in these plants and no differences were found between all mutants and null segregants (Supplementary Fig. S3C, D). The experiment was repeated using the same set of seeds, under the same treatments, and found to have similar results where shoot Cl− accumulation was decreased in amiRNA:*SLAH1* lines (Supplementary Fig. S3E). Under high Cl− supply, amiRNA lines had significantly reduced *AtSLAH1* transcript abundance compared with the null segregants in the same conditions (Supplementary Fig. S4A); however, the *AtSLAH1* expression in the null segregants was reduced by a quarter compared with low Cl− conditions (Fig. 1A and Supplementary Fig. S4A). At the same time there was no difference in shoot Cl− concentration

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**Fig. 1.** Expression level of *AtSLAH1* (A) and *AtSLAH3* (B) treated with control (2 mM NaCl), 50 mM and 100 mM NaCl for 7 days, or 20 μM ± cis-trans-ABA for 4 or 16 h. Arabidopsis (Col-0) were grown in hydroponics for 5 weeks and exposed to NaCl treatment for 7 days. The ABA was applied 4 or 16 h before harvest. Transcripts were detected in the whole root cDNA. Results are presented as means±SEM, *n*=5. The expression levels were normalized to four control genes (AtGAPDH, AtActin2, AtTubulin and AtCyclophilin). Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey’s test (*P*≤0.05); a, b and c represent data groups that are statistically different from each other.
AtSLAH1 modulates shoot Cl\textsuperscript{−} accumulation

between the knockdown plants and controls under high Cl\textsuperscript{−} supply (Supplementary Fig. S4B). This was presumably due to the native downregulation of AtSLAH1 expression by high salt (Figs 1A and 2A and Supplementary Fig. S4A).

Plants constitutively overexpressing AtSLAH1 accumulate high Cl\textsuperscript{−} in the shoot under high Cl\textsuperscript{−} supply

Plants with constitutive overexpression of AtSLAH1 and their null segregants were selected by determining the presence or absence of the AtSLAH1 transgene by PCR of genomic DNA. Relative expression of total AtSLAH1 (consisting of both the native and the transgenic AtSLAH1) was then determined in root tissue by semi-quantitative RT-PCR. AtSLAH1 was found to be highly expressed in both 35S:AtSLAH1 lines generated, whereas the null segregants had less abundant expression (Fig. 3A). When 75 mM NaCl was applied to 35S:AtSLAH1\textsubscript{1}, 35S:AtSLAH1\textsubscript{2} and null segregant lines for 7 days, significantly higher shoot Cl\textsuperscript{−} concentration accumulated in the overexpression lines when compared with the null segregants (P≤0.05), with no difference found between the two independent overexpression lines in shoot Cl\textsuperscript{−} concentration (Fig. 3B). The shoot NO\textsubscript{3}\textsuperscript{−} in both overexpression lines displayed no differences compared with null segregants (Supplementary Fig. S5A). Therefore, the increase in shoot Cl\textsuperscript{−} accumulation resulted in a decrease in shoot NO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} ratio in both overexpression lines under high Cl\textsuperscript{−} conditions (P≤0.05) (Fig. 3C). Both 35S:AtSLAH1 overexpression lines had significantly less whole shoot biomass when compared with the null segregants (P≤0.005) (Fig. 3D).

Under low Cl\textsuperscript{−} supply, the shoot Cl\textsuperscript{−} and NO\textsubscript{3}\textsuperscript{−} concentration of both 35S:AtSLAH1 overexpression lines was not significantly different from each other or the null segregants (Supplementary Fig. S5B, C), and the shoot biomass between all the genotypes was not significantly different (Supplementary Fig. S5D).

Stelar-specific overexpression of AtSLAH1 is correlated with increased shoot Cl\textsuperscript{−} accumulation under high Cl\textsuperscript{−} supply

To further study the function of AtSLAH1 and avoid potential problems caused by non-targeted overexpression in all cell types, root stelar cell-type specific over expression lines were generated following the method outlined by Møller et al. 2009. Two independent lines, named GAL4:AtSLAH1\textsubscript{1} and GAL4:AtSLAH1\textsubscript{2} were grown in hydroponics for 6 weeks before being supplied with 2 or 75 mM NaCl for a further 7 days. Under high Cl\textsuperscript{−} conditions, both cell-specific overexpression lines had greater accumulation of Cl\textsuperscript{−} within the shoot; AtSLAH1 expression and shoot Cl\textsuperscript{−} accumulation were again positively correlated (R\textsuperscript{2}=0.5, P≤0.01) (Fig. 4A). As with the constitutively overexpressing AtSLAH1 plants, high salt treatment led to greater shoot Cl\textsuperscript{−} accumulation and no alteration in shoot concentration of Na\textsuperscript{+}, K\textsuperscript{+} or NO\textsubscript{3}\textsuperscript{−} in the cell-specific overexpression lines compared to the null

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**Fig. 2.** Under low Cl\textsuperscript{−} conditions, amiRNA:AtSLAH1 mutants had significantly reduced expression levels of AtSLAH1 and reduced shoot Cl\textsuperscript{−} compared with null segregants. Plants were grown hydroponically for 6 weeks in BNS containing 2 mM NaCl (low Cl\textsuperscript{−} conditions). (A) AtSLAH1 expression in roots of all amiRNA:AtSLAH1 mutants (amiRNA:AtSLAH1\textsubscript{1}, 2, 3 and 4) and null segregants (nulls). (B) Shoot Cl\textsuperscript{−} accumulation of amiRNA:AtSLAH1 mutants and nulls under low Cl\textsuperscript{−} conditions. (C) Correlation between transcript level of AtSLAH1 and shoot Cl\textsuperscript{−} concentration. (D) The shoot NO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} ratio in all amiRNA:AtSLAH1 mutant and null segregant lines grown under low Cl\textsuperscript{−} conditions. Results are mean±SEM (n=8), except (C), which is ±SEM. Statistical differences determined by one-way ANOVA and Tukey’s test (P≤0.005); a and b represent statistically significant differences between data groups.
segregant lines (Supplementary Fig. S6A, B, C). Furthermore, the shoot biomass of the cell-specific overexpression lines was reduced under high salt treatment compared with the null segregants (Fig. 4B).

Under low Cl\textsuperscript{−} conditions, no significant differences in shoot Cl\textsuperscript{−} or NO\textsubscript{3}\textsuperscript{−} accumulation were identified between stelar-specific AtSLAH1 overexpression lines and the null segregants (Supplementary Fig. S7A, B), but significantly less shoot Cl\textsuperscript{−} was accumulated in these plants in low Cl\textsuperscript{−} than when in high Cl\textsuperscript{−} treatment (Fig. 4A). Under low Cl\textsuperscript{−} conditions, the shoot biomass of both stelar-specific AtSLAH1 overexpression lines was not significantly different from the null segregants (Supplementary Fig. S7C).

**Fig. 3.** Under high Cl\textsuperscript{−} conditions, 35S:AtSLAH1 overexpression lines accumulated higher shoot Cl\textsuperscript{−} and showed reduced NO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} ratio compared with null segregants (nulls). Plants were grown hydroponically in BNS until 6 weeks old and then exposed to BNS containing 75 mM NaCl (high Cl\textsuperscript{−} conditions) for 7 days. (A) Semi-quantitative RT-PCR of 35S:AtSLAH1 overexpression lines and nulls. (B) Shoot Cl\textsuperscript{−} concentration under high Cl\textsuperscript{−} conditions. (C) Shoot NO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} ratio. (D) Whole shoot biomass (fresh weight) as measured after high Cl\textsuperscript{−} treatment. Results are mean±SEM (n=6). Statistical differences determined by one-way ANOVA and Tukey’s test (P≤0.05); a and b represent statistically significant differences between data groups.

**Fig. 4.** Stelar cell type-specific overexpression of AtSLAH1 in E2586 significantly increased shoot Cl\textsuperscript{−} accumulation and reduced shoot biomass under high Cl\textsuperscript{−} conditions. (A) Correlation between shoot Cl\textsuperscript{−} accumulation and relative expression of AtSLAH1 in GAL4:AtSLAH1 overexpression lines under high Cl\textsuperscript{−} (75 mM NaCl) supply. Open circles and solid line: GAL4:AtSLAH1_1; open triangle and dash line: GAL4:AtSLAH1_2. Plants were grown hydroponically in BNS for 6 weeks and then exposed to BNS containing 75 mM NaCl (high Cl\textsuperscript{−} conditions) for 7 days. Correlation between shoot Cl\textsuperscript{−} concentration and the abundance of AtSLAH1 in GAL4:AtSLAH1_1 (R\textsuperscript{2}=0.5805, P≤0.005, significant deviation from zero), GAL4:AtSLAH1_2 (R\textsuperscript{2}=0.5395, P≤0.005, significant deviation from zero). (B) Whole shoot biomass (fresh weight) as measured after high Cl\textsuperscript{−} treatment. Results are mean±SEM (n=6). Statistical differences determined by one-way ANOVA and Tukey’s test (P≤0.05); a and b represent statistically significant differences between data groups.

AtSLAH3 overexpression increases the shoot NO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} ratio under high and low Cl\textsuperscript{−}

To compare the effects of AtSLAH1 misexpression with that of a close homologue known to have a preference for NO\textsubscript{3}\textsuperscript{−} transport, we examined the phenotype of plants constitutively overexpressing AtSLAH3. In contrast to the greater Cl\textsuperscript{−} accumulation we observed in shoots of AtSLAH1-overexpressing
AtSLAH1 is likely to require additional co-factors to be active in *X. laevis* oocytes

*AtSLAH1* was expressed in *X. laevis* oocytes in an attempt to examine whether it could directly catalyse the transport of Cl\(^-\) (Supplementary Materials and methods). No functional activity could be detected when *AtSLAH1* was expressed by itself (Supplementary Fig. S9). The *AtSLAH1* homologue, *AtSLAC1*, was also found to be electrically silent in oocytes when expressed by itself, but when expressed with *sucrose non-fermenting-1-related protein kinase 2.6* (SnRK2.6) *AtSLAC1* carried currents (Geiger *et al.*, 2009). To investigate whether a similar regulatory process was also required to trigger anion transport by *AtSLAH1* in heterologous systems, *AtSnRK2.2* and *AtSnRK2.3* (root localized homologues of *AtSnRK2.6* that have their expression regulated by ABA; *Yoshida et al.*, 2006; *Fuji and Zhu*, 2009; *Nakashima et al.*, 2009) were co-injected with *AtSLAH1* in *X. laevis* oocytes (Supplementary Fig. S9F–H) but this resulted in no consistent activation of current, suggesting additional cofactors that regulate SLAH1 function still need to be identified.

**Discussion**

**AtSLAH1 meets the predicted characteristics for a gene controlling Cl\(^-\) loading into the root xylem**

Previous studies showed that *AtSLAH1* belongs to the *AtSLAC1* family; SLAC1 and SLAH3 underpin components of the slow type (S-type) anion conductance involved in anion efflux across the PM of stomatal guard cells in response to CO\(_2\) and O\(_3\) (Negi *et al.*, 2008; *Vahisalu et al.*, 2008; Geiger *et al.*, 2011; Demir *et al.*, 2013). The guard cell PM S-type anion conductance was found to be permeable to malate, Cl\(^-\) and NO\(_3^-\) and its activation triggered by ABA (Schroeder and Hagiwara, 1989; Hedrich, 2012). *AtSLAH3* and another family member, *AtSLAH2*, have been predicted to load NO\(_3^-\) into the root xylem (Maierhofer *et al.*, 2014; Zheng *et al.*, 2015). Whilst *AtSLAH1* is not usually expressed in guard cells it could complement the wild-type guard cell function of the *slac1* knockout when ectopically expressed, indicating it may encode or regulate a functional channel (Negi *et al.*, 2008; *Vahisalu et al.*, 2008); however, its true physiological functions are yet to be deciphered. Here, we confirmed that *AtSLAH1* was highly expressed in the Arabidopsis root and its expression was down-regulated strongly by ABA and NaCl treatment (Fig. 1). Previously *AtSLAH1* was shown to be expressed in the root stele and pericycle of Arabidopsis roots and present on the PM (Brady *et al.*, 2007; Gifford *et al.*, 2008; Negi *et al.*, 2008; Supplementary Fig. S2). As the stelar-localized PM conductances capable of loading anions into the root xylem (and consequently the shoot) have been observed to be down-regulated by ABA (Gillham and Tester, 2005) this suggests that *AtSLAH1* could be involved in significant Cl\(^-\) loading of the root xylem. In contrast, we found that *AtSLAH3* transcript abundance was not reduced by salt or down-regulated by ABA (Fig. 1B). This coupled to the higher NO\(_3^-)/Cl\(^-\) ratio of *AtSLAH3*-overexpressing plants (Supplementary Fig. S8E and F) suggests it does not, by itself, contribute to a significant proportion of Cl\(^-\) accumulation in the shoot.

**AtSLAH1 regulates Arabidopsis shoot Cl\(^-\) accumulation**

As the ‘Atslah1’ T-DNA insertion mutant from the European Arabidopsis Stock Centre retained expression of *SLAH1*, we generated amiRNA lines that had reduced expression of *AtSLAH1* (Fig. 2A). Under low Cl\(^-\) supply (2 mM NaCl), all *AtSLAH1* amiRNA lines had lower accumulation of Cl\(^-\) but not of NO\(_3^-\) in the shoot (Fig. 2A, B and Supplementary Fig. S3A). There was also a strong positive correlation between *AtSLAH1* expression levels and shoot Cl\(^-\) (Fig. 2C), which suggests that *AtSLAH1* might play an important role in regulating Cl\(^-\) transport from root to shoot by affecting net loading of xylem vessels in the root. Whilst the shoot NO\(_3^-\) concentration did not significantly alter when compared with the null segregants (Supplementary Fig. S3A), reduced *AtSLAH1* expression did lead to an increased shoot NO\(_3^-)/Cl\(^-\) ratio due to a lower amount of Cl\(^-\) in the shoot (Fig. 2D).

Shoot Cl\(^-\) concentration was also examined in all amiRNA: *AtSLAH1* mutants exposed to high salt stress. No shoot Cl\(^-\) concentration differences were found between mutants and the null segregants under these conditions (Supplementary Fig. S4B). *AtSLAH1* expression is naturally decreased under high concentrations of NaCl (Fig. 1A). Therefore it is reasonable to suggest that the unchanged shoot Cl\(^-\) concentration in these plants was probably due to the endogenous down-regulation of *AtSLAH1* caused by high salinity. Therefore, the results of *AtSLAH1* overexpression lines might be expected to be more instructive for determining *AtSLAH1* function under high salt conditions.

In 35S:*AtSLAH1* overexpression lines we observed significantly increased shoot Cl\(^-\) accumulation compared with null segregants when grown under high Cl\(^-\) (75 mM NaCl) (Fig. 3B); this is again consistent with *AtSLAH1* being involved in xylem Cl\(^-\) loading. No difference in shoot NO\(_3^-\), K\(^+\) or Na\(^+\) accumulation was observed between 35S:*AtSLAH1* overexpression lines and null segregants under high Cl\(^-\) supply (Supplementary Fig. S5A, E, F) indicating that the role of *AtSLAH1* is specific for Cl\(^-\). This translated into a reduced NO\(_3^-)/Cl\(^-\) ratio in these lines compared with nulls (Fig. 3C). In overexpression lines, there was a concomitant decrease in shoot biomass compared with null segregant lines (Fig. 3C), suggesting that the level of Cl\(^-\) accumulated, and the reduction in NO\(_3^-)/Cl\(^-\) ratio over this time period is sub-optimal for growth. In many studies, the shoot K\(^+\)/Na\(^+\) ratio...
is widely used to evaluate the plant’s salt tolerance: a higher K+/Na+ ratio value normally indicates a better salinity tolerance (Tester and Davenport, 2003). Due to the antagonism between Cl- and NO3− transport and the key roles of NO3− in plant metabolism, it is reasonable to suggest that mechanisms that maintain high NO3−/Cl− ratios might also be beneficial for improving salt tolerance.

These effects on shoot Cl− accumulation, shoot NO3−/Cl− ratio and shoot biomass were replicated in AtSLAH1 root stelar cell-specific overexpression lines in high Cl− conditions (in the cell types in which AtSLAH1 is ordinarily expressed (Brady et al., 2007; Gifford et al., 2008; Negi et al., 2008) (Fig. 4)). This indicates that constitutive overexpression of AtSLAH1 did not result in significant pleiotropic responses, which may be to do with the need for an unknown interacting partner in its native cell type for AtSLAH1 to be functional. What is important to note is that in AtSLAH1 constitutively overexpressing plants and in the root stelar specific AtSLAH1 overexpression lines in low Cl− growth conditions, there was no growth phenotype compared with the null segregants (Figs 3 and 4). This linked to the fact that other ion contents (K+, Na+ or NO3−) were not altered in any conditions (Supplementary Figs S5 and S6), demonstrates that the growth of AtSLAH1-overexpressing plants was not altered by overexpression of the AtSLAH1 protein per se. Rather the inhibition of growth seen for AtSLAH1-overexpressing plants in high Cl− was specifically due to the additional accumulation of Cl− in the shoot (Figs 3 and 4).

Chloride accumulation in the shoot is a multigenic trait (White, 2001). We have shown that AtSLAH1 is likely to make up a component of this, as it has a significant effect on shoot Cl− accumulation (~20–40% in various conditions), which suggests that other Arabidopsis anion transport proteins might be involved in root to shoot Cl− transport. Recently, NFP2.4, a transport protein localized to the PM of stelar cells, was found to be important for Cl− but not for NO3− accumulation in shoots (Li et al., 2016). AtCCC has also been shown to have an impact on shoot Cl− accumulation (Colmenero-Flores et al., 2007; Henderson et al., 2015). However, AtCCC is predominantly localized to the Golgi and trans-Golgi network and so is unlikely to have a direct role in net loading of Cl− into the xylem (Henderson et al., 2015). Other candidates include transporters designated as NO3− permeable, but which may also transport some Cl−, such as NRT1.5/NPF7.3, NRT1.8/NPF7.2 and SLAH3 (Lin et al., 2008; Li et al., 2010). A transcriptional comparison between the roots of good and poor Cl−-excluding grapevine rootstocks suggested further candidate genes for this multigenic trait including aluminium-acclimated malate transporters (ALMT), chloride channels (CLC) and their putative activating kinases (Henderson et al., 2014). However, the true involvement of these candidate proteins requires that their substrates are resolved by functional assays. Therefore, the observed phenotypes in any single gene mutant are likely to be complicated by other functional proteins involved in Cl− accumulation in the shoot.

AtSLAH1 is likely to require unknown interacting proteins to function

No anion-mediated currents were identified when AtSLAH1 cRNA was injected into X. laevis oocytes (Supplementary Fig. S9C, D). AtSLAC1, AtSLAH2 and AtSLAH3 are known to require protein kinases to be functional in oocytes (Geiger et al., 2009; Vahisalu et al., 2010; Brandt et al., 2012; Demir et al., 2013; Gutermuth et al., 2013; Maierhofer et al., 2014). For instance, when SLAC1 was expressed in X. laevis oocytes no clear anion currents were generated (Vahisalu et al., 2008); however, co-expression with the protein kinase SnRK2.6 in oocytes was found to activate SLAC1 by phosphorylation of multiple serines in the SLAC1 hydrophilic N-terminal sequence (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010). This evidence suggests that a similar regulatory component is important for activating the S-type anion channels and may be required for SLAH1 activity. As SnRK2.6 has low expression in roots we concentrated on the root expressed members of the ABA-activated SNF1-related protein kinases 2, SnRK2.2 and SnRK2.3, which are both involved in ABA signalling pathways in roots (Yoshida et al., 2006; Fuji and Zhu, 2009; Nakashima et al., 2009). To identify whether a phosphorylation process initiated by a protein kinase was required to activate SLAH1 in oocytes, SnRK2.2/2.3 was co-injected with AtSLAH1 into oocytes (Supplementary Fig. S9G–J). However, no activity was observed. Therefore, it is likely that AtSLAH1 requires additional factors for it to be active, or that it is itself a regulator of transport through its interaction with another transport protein.

Conclusions

Manipulating AtSLAH1 expression level in Arabidopsis resulted in significant alterations in shoot Cl− concentrations suggesting AtSLAH1 is involved in Cl− xylem loading in roots and the regulation of Cl− accumulation in the shoot in response to salt stress. In contrast, overexpression of AtSLAH3 resulted in relatively greater NO3− content of the shoot under low and high Cl− treatments. In the present work, heterologous expression studies were unable to distinguish whether AtSLAH1 acts directly as a transport protein or transport regulator. As AtSLAH1 expression decreases under salinity and ABA, but AtSLAH3 is still expressed, the relative capacity for roots to deliver NO3− to the shoot is increased under saline conditions but the capacity for Cl− loading is reduced. This will serve to maximize important NO3− delivery to the shoot despite the increased competition from Cl− during salinity stress. Therefore, it appears likely that AtSLAH1 and AtSLAH3 act in tandem to regulate NO3− and Cl− loading to the shoot and are both targets for manipulation in crops to improve salinity tolerance.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. AtSLAH1 is still expressed in the slah1 homozygous T-DNA insertion line FLAG_336C06.
AtSLAH1 modulates shoot Cl\textsuperscript{−} accumulation

Figure S2. The transcript level changes of \textit{AtSLAH1} and \textit{AtSLAH3} upon NaCl or ABA treatment.

Figure S3. Under low Cl\textsuperscript{−} conditions, the shoot NO\textsubscript{3}\textsuperscript{−}, Na\textsuperscript{+}, K\textsuperscript{+} concentrations and biomass were detected in all amiRNA:AtSLAH1 mutants and null segregants (nulls).

Figure S4. Transcript abundance of \textit{AtSLAH1} amiRNA containing lines (T\textsubscript{2}) and shoot Cl\textsuperscript{−} concentration under high Cl\textsuperscript{−} stress.

Figure S5. The shoot NO\textsubscript{3}\textsuperscript{−}, Cl\textsuperscript{−} concentrations and shoot biomass were detected under low and high Cl\textsuperscript{−} supply in both 35S:AtSLAH1\textsubscript{1} and 35S:AtSLAH1\textsubscript{2}, and null segregants (nulls).

Figure S6. Under high Cl\textsuperscript{−} conditions, the shoot Na\textsuperscript{+}, K\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−} concentrations were detected in all GAL4:AtSLAH1 overexpression lines and null segregants (nulls).

Figure S7. Under low Cl\textsuperscript{−} conditions, shoot Cl\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−} concentrations and whole shoot biomass were detected in all GAL4:AtSLAH1 overexpression lines and null segregant.

Figure S8. The shoot NO\textsubscript{3} and Cl\textsuperscript{−} concentrations were detected under low and high Cl\textsuperscript{−} supply in both 35S:AtSLAH3\textsubscript{1} and 35S:AtSLAH3\textsubscript{2}, and null segregant (nulls) lines.

Figure S9. Electrophysiological characterization of \textit{AtSLAH1} in \textit{X. laevis} oocytes.

Table S1. Primers used for generating amiRNA:AtSLAH1 constructs, for screening homozygous \textit{Atslah1}\textsuperscript{T-DNA} mutant lines and for cloning \textit{AtSLAH1/AtSLAH3} from Arabidopsis.

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References


