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SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling

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Abstract

Stomatal pores, formed by two surrounding guard cells in the epidermis of plant leaves, allow influx of atmospheric carbon dioxide in exchange for transpirational water loss. Stomata also restrict the entry of ozone—an important air pollutant that has an increasingly negative impact on crop yields, and thus global carbon fixation¹ and climate change². The aperture of stomatal pores is regulated by the transport of osmotically active ions and metabolites across guard cell membranes^{3,4}. Despite the vital role of guard cells in controlling plant water loss^{3,4}, ozone sensitivity^{1,2} and CO₂ supply^{2,5–7}, the genes encoding some of the main regulators of stomatal movements remain unknown. It has been proposed that guard cell anion channels function as important regulators of stomatal closure and are essential in mediating stomatal responses to physiological and stress stimuli^{3,4,8}. However, the genes encoding membrane proteins that mediate guard cell anion efflux have not yet been identified. Here we report the mapping and characterization of an ozone-sensitive *Arabidopsis thaliana* mutant, *slac1*. We show that *SLAC1* (*SLOW ANION CHANNEL-ASSOCIATED 1*) is preferentially expressed in guard cells and encodes a distant homologue of fungal and bacterial dicarboxylate/malic acid transport proteins. The plasma membrane protein SLAC1 is essential for stomatal closure in response to CO₂,

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Author Contributions T.V., H.K. and Y.-F.W. contributed equally to this work. J.K. and H.K. designed the experiments in Figs 1 and 2. A.L., H.K. and T.V. identified the *SLAC1* gene. T.V. and M.B. performed the expression, complementation and subcellular localization analyses in Fig. 1 and Supplementary Fig. 5. H.K. and H.M. performed experiments in Fig. 2. H.K. performed experiments in Supplementary Figs 1 and 2. R.D. designed and performed experiments in Fig. 3b, c and Supplementary Fig. 6b. J.I.S. and J.K. designed experiments in Figs 3a and d, and 4, and Supplementary Figs 6a, 7, 8 and 9. W.-Y.C. and G.V. performed experiments in Fig. 3d and Supplementary Fig. 6a. N.N. performed experiments in Fig. 3a and Supplementary Fig. 7. Y.-F.W. performed experiments in Fig. 4 and Supplementary Figs 8 and 9. J.K. and J.I.S. wrote the paper. All the authors discussed the results, and commented on and edited the manuscript.

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abscisic acid, ozone, light/dark transitions, humidity change, calcium ions, hydrogen peroxide and nitric oxide. Mutations in *SLAC1* impair slow (S-type) anion channel currents that are activated by cytosolic Ca^{2+} and abscisic acid, but do not affect rapid (R-type) anion channel currents or Ca^{2+} channel function. A low homology of *SLAC1* to bacterial and fungal organic acid transport proteins, and the permeability of S-type anion channels to malate⁹ suggest a vital role for *SLAC1* in the function of S-type anion channels.

Stomatal aperture is regulated by light, plant water status, CO_2 concentration, relative air humidity, and among other stresses, drought and ozone (O_3)^{3,4}. A number of signalling compounds, including abscisic acid (ABA), reactive oxygen species (ROS), nitric oxide (NO) and Ca^{2+} ions are involved in the regulation of stomatal aperture^{3,4}. Adjustment of stomatal apertures is achieved by controlled transport of osmotically active ions and organic metabolites, including potassium (K^+), chloride (Cl^-) and malate across guard cell membranes^{3,8,10,11}, resulting in changes in osmotic potential. Anion channels have been proposed to function as central regulators of stomatal closure^{8,11} by mediating anion efflux and causing membrane depolarization, which controls K^+ efflux through K^+ channels. So far, none of the candidates for plant anion channels — the plant homologues to the animal CLC chloride channels — has been localized to the plasma membrane¹⁰, and the first plant CLC channel that was functionally characterized encodes a central vacuolar proton/nitrate exchanger¹², rather than an anion channel. Thus, despite their proposed importance in several physiological and stress responses in plants^{8,10,11}, the molecular identity of the guard cell plasma membrane proteins that mediate anion channel activity has remained unknown.

In a mutant screen for O_3 sensitivity, a series of *Arabidopsis* ethyl methanesulphonate (EMS) mutants called *radical-induced cell death* (*rcd*) was identified^{13,14}. One of them, a recessive mutant originally referred to as *rcd3* (ref. 14) and here renamed *slac1* (*slow anion channel-associated 1*), showed constitutively higher stomatal conductance than the wild type (Columbia, Col-0) (Fig. 1a). Interestingly, both rapid transient¹⁵ and long-term O_3 -induced decreases in stomatal conductance were abolished in *slac1* (Fig. 1a). Water loss from excised *slac1* leaves resulted in 70–80% fresh weight loss after 90 min, whereas in the wild type, fresh weight loss was only 30% after 90 min (Fig. 1b). These differences in fresh weight loss were not a result of variation in stomatal number because *slac1* and wild-type leaves have similar stomatal density (Supplementary Fig. 1). Microarray analyses using messenger RNAs from 3-week-old rosette leaves did not reveal any significant differences in gene expression between *slac1* and the wild type when grown under optimal conditions. Furthermore, no other phenotypic differences have been observed between *slac1* and the wild type. Together these data suggested that the defect in *slac1* lies in defective stomatal regulation and that the O_3 damage of *slac1* leaves (Supplementary Fig. 2) is a result of increased O_3 flux into leaves through more open stomata.

The *slac1-1* mutation was identified in the gene At1g12480 by a combination of mapping, candidate gene expression in guard cell microarrays, and analyses of transfer DNA (T-DNA) insertion mutants (see Supplementary Information). *SLAC1* encodes a predicted membrane protein of 556 amino acids with a calculated molecular weight of 63.2 kDa and a predicted isoelectric point of 9.58. *SLAC1* has hydrophilic amino- and carboxy-terminal tails (189 and 60 amino acids, respectively) and 10 predicted transmembrane helices (Fig. 1c, Supplementary Fig. 3), which contain a C4-dicarboxylate transporter/malic acid transport protein domain (InterPro: IPR004695) defined from the *Escherichia coli* TehA and *Schizosaccharomyces pombe* Mae1 proteins. Mae1 is involved in malate uptake¹⁶. TehA and Mae1 lack the long hydrophilic tail present in the N terminus of *SLAC1*, but show a weak, 15–20% amino-acid identity over the transmembrane region with *SLAC1*.

(Supplementary Fig. 4a). SLAC1 shows no homology to the aluminium-activated malate transporters that function in plant aluminium resistance¹⁷. Homozygous T-DNA insertion lines (SALK_099139 and SALK_137265, referred to as *slac1-3* and *slac1-4*, respectively; Fig. 1c) both showed similar recessive inheritance, and exhibited similar fresh weight loss from excised leaves as *slac1-1* (Fig. 1b). A genomic copy of *SLAC1* complemented the mutant phenotype in stably transformed *slac1-1* (Fig. 1b).

SLAC1 belongs to a small family of five proteins in *Arabidopsis*. Three of the proteins, including SLAC1, have a long hydrophilic N-terminal tail, whereas two have only the transmembrane domains. Rice has nine orthologous proteins. The SLAC1 protein is more similar to its rice orthologue Os04g48530 than to the four other *Arabidopsis* SLAC1 homologues (Supplementary Fig. 4a, b). The transmembrane domains of *Arabidopsis* and rice SLAC1 homologues and orthologues have several highly conserved amino acids. However, SLAC1 and Os04g48530 also differ from the rest of the proteins in several amino-acid residues (Supplementary Fig. 4a). For example, the amino acid that is mutated in *slac1-1* is a serine in SLAC1 and Os04g48530, whereas other family members have an alanine residue in the same position. The serine mutated in *slac1-1* is surrounded by three conserved threonine residues, suggesting that this region is significant for either the structure, function or regulation of the protein. Additionally, the predicted intracellular loops between the transmembrane domains have several conserved, positively charged amino-acid residues (Supplementary Figs 3, 4a), also suggesting functional significance.

When 1,582 base pairs (bp) of genomic sequence upstream of the *SLAC1* translation start were fused to the reporter gene *uidA*, the resulting β -glucuronidase (GUS) activity in transgenic plants was localized predominantly to guard cells (Fig. 1d), and occasionally to the vascular strands close to the leaf margins (Fig. 1e). No GUS activity was detected in other parts of the plants. Expression data at the Genevestigator database¹⁸ and comparison of gene expression between guard cell and mesophyll cell microarrays also suggest strong preferential guard cell expression of *SLAC1*.

To study the subcellular location of the SLAC1 protein, green fluorescence protein (GFP) fused to the SLAC1 C terminus was transiently expressed in onion epidermal cells (Fig. 1f–k) and in tobacco protoplasts (Supplementary Fig. 5). Fluorescence and confocal imaging showed that in onion epidermal cells, fluorescence from the SLAC1::GFP fusion protein (Fig. 1f) and the membrane-specific stain FM 4-64 (Fig. 1g) colocalized in merged images (Fig. 1h). GFP fluorescence was observed between the cell wall and the nucleus (Fig. 1j; Supplementary Movie), and was connected to the cell wall through Hechtian strands in plasmolysed cells (Fig. 1k), correlating with plasma membrane localization. Expression in tobacco protoplasts showed results that are consistent with plasma membrane localization (Supplementary Fig. 5).

Stomatal aperture is under environmental and hormonal control. We analysed stimulus responses in stomatal conductance by comparing intact¹⁵ *slac1* with wild-type plants. Stomatal conductance in *slac1* was about 1.5-fold higher during the light period (Fig. 2a). Also, the decline in stomatal conductance at the beginning of the dark period took more than 1 h longer in *slac1* compared with the wild type (Fig. 2a). Light/dark transitions during the normal light period caused rapid changes in stomatal conductance in the wild type, whereas *slac1* showed a slow and modest response (Fig. 2b). *slac1* exhibited a much slower response than the wild type to a decrease in the relative air humidity (Fig. 2c), which is known to cause a rapid reduction of stomatal conductance¹⁹. Doubling of [CO₂] from 400 p.p.m. to 800 p.p.m. reduced stomatal conductance effectively in the wild type, whereas *slac1* showed no responses (Fig. 2d). Thus, *slac1* stomata show only a slow and modest response to

changes in light and air humidity, and are completely insensitive to O₃ stress (Fig. 1a) and elevated [CO₂] (Fig. 2d).

The concentration of the plant stress hormone ABA increases under drought and induces stomatal closure through second messengers, including ROS, cytosolic Ca²⁺ and NO^{20–22}. We measured stomatal responses to ABA, hydrogen peroxide (H₂O₂), NO and repetitive Ca²⁺ pulses (Fig. 3). Stomata of *slac1* mutants showed a strong insensitivity to ABA (Fig. 3a and Supplementary Fig. 6a). Similarly, they showed significantly reduced responses to H₂O₂ (Fig. 3b) and the NO donor sodium nitroprusside (SNP) (Fig. 3c). Transient addition and removal of Ca²⁺ to the extracellular solution bathing leaf epidermides, while shifting the K⁺ equilibrium potential, allows experimental imposition of defined intracellular Ca²⁺ transients in guard cells, resulting in stomatal closure^{23–25}. Four repetitive 5-min pulses of 1 mM external Ca²⁺ were applied (Fig. 3d; top inset; Supplementary Fig. 7). The imposed intracellular Ca²⁺ ([Ca²⁺]_i) oscillation pattern of *slac1-1* guard cells was similar to that of the wild type (Supplementary Fig. 7). The average amplitudes of imposed [Ca²⁺]_i transients and the integrated total [Ca²⁺]_i increases per period were statistically similar in wild-type and *slac1-1* guard cells (see Supplementary Information). Imposed [Ca²⁺]_i transients caused the typical downstream Ca²⁺-induced reactive and programmed^{23,24} stomatal closure in the wild type, whereas the response was greatly impaired in *slac1-1* and *slac1-3* (Fig. 3d). Thus *slac1* mutant guard cells do not abrogate imposed cytosolic Ca²⁺ oscillations, but show a strong impairment in downstream Ca²⁺ oscillation-induced stomatal closing.

The activation of S- and R-type anion efflux channels, both of which can transmit Cl[−] and malate efflux from guard cells^{8,9,11}, is proposed to decrease guard cell osmotic potential, leading to stomatal closure^{3,4,8,10,11}. This is consistent with Cl[−] and malate efflux occurring in response to ABA^{26,27}. We therefore applied whole-cell patch clamp techniques to characterize the functioning of S-type and R-type anion channel activities. In wild-type guard cells, elevated cytosolic Ca²⁺ (2 μM) activated ion currents that were selective for Cl[−] over caesium ions (Cs⁺) (*n* = 16 guard cells) and showed a relative permeability ratio for malate to chloride anions of 0.125 (*n* = 12 guard cells), consistent with previous anion selectivity analyses of S-type anion channel currents⁹ (Supplementary Fig. 8).

S-type anion currents were readily recorded in wild-type guard cells (Fig. 4a, d). However, only very small combined background whole-cell membrane currents and patch-clamp seal currents were observed in *slac1-1* and *slac1-3* guard cells (Fig. 4b–d). R-type anion currents¹¹ were activated as described^{25,28}. Interestingly, no significant differences in R-type anion currents between wild-type and *slac1* guard cells were observed (Fig. 4e, f). Similarly, ABA activation of Ca²⁺-permeable 'I_{Ca}' channel currents²¹ was not disrupted in *slac1* guard cells (Supplementary Fig. 9). However, when ABA activation of S-type anion channels was analysed, *slac1* mutants showed only small whole-cell currents (Fig. 4h–j), whereas S-type anion currents were recorded in wild-type guard cells (Fig. 4g, j).

Continuing increases in ozone concentrations in the troposphere owing to human activities are predicted to have a negative affect on crop yields and global carbon sinks in the future^{1,2}. The ozone sensitivity of *slac1* leaves (Supplementary Fig. 2), the predominant guard cell expression of *SLAC1* (Fig. 1d, e) and abolishment of O₃-induced stomatal closure in *slac1* mutants (Fig. 1a) together provide direct genetic evidence for the importance of O₃ sensing in guard cells for plant O₃ tolerance. Only a few plant mutants are known that show CO₂ insensitivity^{5,6} or a constitutive high CO₂ response⁷ in stomatal movements, but no recessive CO₂-insensitive mutant gene has been isolated so far. All *slac1* alleles are recessive and show a complete lack of high CO₂-induced stomatal closure (Fig. 2), illustrating that the SLAC1 protein is a central positive mediator of CO₂-induced stomatal closure.

Experiments with ABA, ROS, NO and Ca^{2+} suggest that SLAC1 is an essential protein functioning downstream of these messengers in mediating stomatal closure (Figs 3, 4 and Supplementary Figs 6, 7, 9). The phenotype of *slac1* differs from the ATP-binding cassette transporter mutant, *atmrp5*, which shows partial repression of ABA-induced stomatal closure, partial S-type anion current activity and impaired Ca^{2+} channel activation²⁹. The strong impairment in S-type anion channel and normal Ca^{2+} channel activity in *slac1* guard cells is consistent with SLAC1 being more closely associated with S-type anion channels than is AtMRP5, and provides direct genetic evidence for the model that these anion channels function as a central control mechanism for stomatal closure⁸.

R-type anion channel activity was not disrupted in *slac1* guard cells (Fig. 4e, f), providing genetic evidence for a molecular separation of the membrane proteins required for S- and R-type anion channels. It remains possible that these anion channel types share other protein subunits³⁰. R-type channels may be responsible for the slow stomatal conductance decrease observed in response to light/dark transitions and decrease in relative humidity (Fig. 2a–c).

The data presented demonstrate that *SLAC1* encodes an essential subunit for S-type anion channel function or regulation. The low homology of SLAC1 to bacterial and fungal organic acid transporters indicates a possible role for SLAC1 in contributing to formation of an anion-transporting pore. Further research on SLAC1 and its homologues should increase the general understanding of plasma-membrane anion channel structure and regulation in plants.

METHODS SUMMARY

Three- to six-week-old *A. thaliana* plants grown in a controlled environment were used. *slac1-1* was isolated from an O_3 -sensitivity mutant screen¹³. The mapping population was generated by outcrossing to *Ler*, and an impaired water-loss phenotype was used as a mapping trait. For water-loss analyses, the weight of the detached leaves was followed. Whole-plant stomatal conductance responses to O_3 , light/dark transitions, elevated CO_2 and lowered humidity were measured using the *Arabidopsis* whole-rosette gas-exchange system¹⁵. For GUS activity and complementation analyses, transgenic *SLAC1* promoter-driven *GUS* expression lines and complementation lines with *SLAC1* genomic DNA were analysed. For transient gene-expression studies, a SLAC1::GFP fusion protein under the control of a 35S promoter was delivered into onion epidermides by particle bombardment, and to tobacco protoplasts by electroporation. Images were acquired by confocal microscopy. For stomatal responses to H_2O_2 , NO and ABA, stomatal apertures were measured from extracted epidermal fragments after pre-incubation of leaves in opening buffer. Stomatal responses to Ca^{2+} transients and Ca^{2+} imaging experiments were analysed in intact leaf epidermides by imposing extracellular calcium pulses^{23,25}. For electrophysiological analyses, *Arabidopsis* guard cell protoplasts were isolated enzymatically, and Ca^{2+} activation of S- and R-type anion currents and ABA activation of S-type anion and $\text{I}_{\text{Ca}} \text{Ca}^{2+}$ currents were recorded as described^{25,29}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Membrane protein SLAC1 controls leaf ozone and water-loss responses

a, Stomatal conductance ($n = 10, \pm \text{s.e.m.}$) of *slac1-1* and wild-type plants after onset of 200 p.p.b. ozone, indicated by arrows. **b**, Weight loss from detached leaves of wild type (WT), *slac1* alleles and *slac1-1* complemented with the *SLAC1* gene ($n = 5, \pm \text{s.e.m.}$). **c**, Membrane spanning hydrophobic regions in SLAC1 protein and the location of mutant alleles. **d, e**, GUS activity in *SLAC1* promoter *uidA* reporter lines. **f**, *SLAC1::GFP* translational fusion expressed in onion epidermal cells. **g**, Area as in **f**, membranes stained with FM4-64. **h**, Overlay of **f** and **g**. **i**, Light micrograph of **h**. **j**, Overlay of **h**, and **i**. **k**, *SLAC1::GFP* translational fusion in plasmolysed onion epidermal cells renders the Hechtian strands attaching the plasma membrane to the cell wall visible. Scale bars: **d–j**, 100 μm ; **k**, 50 μm .

Figure 2. Mutations in *SLAC1* impair stomatal responses to changes in environment

a, Diurnal dark/light stomatal conductance response in *slac1* and wild-type plants with $\pm$ s.e.m. ($n = 3$). **b**, Time courses of stomatal responses to changes in light intensity. **c**, Time courses of stomatal response to changes in air humidity. **d**, Time courses of stomatal response to changes in CO_2 concentration. Stomatal responses were monitored with an *Arabidopsis* whole-rosette gas-exchange system¹⁵, and values in **b–d** were normalized to conductances at 0 min and represent averages ($\pm$ s.e.m.) of four rosettes.

Figure 3. Impaired stomatal responses to ABA, H_2O_2 , NO and Ca^{2+} in *slac1*

a, Time-course experiments of ABA-induced stomatal closure. ABA (1 μ M) was added at time = 0 ($n = 3$ experiments, 28, 23 and 57 stomata for *slac1-1*, *slac1-3* and wild type, respectively). Stomatal apertures at time = 0 (100%) corresponded to average stomatal apertures of 2.82 ± 0.16 μ m (wild type), 2.88 ± 0.12 μ m in *slac1-1* and 3.23 ± 0.08 μ m in *slac1-3*. **b**, **c**, Time course of stomatal closure induced by H_2O_2 (100 μ M) (**b**) and NO (derived from 50 μ M SNP) (**c**). $n = 3-5$ independent experiments, 20 stomata per experiment. **d**, Impairment in stomatal closure in response to four transient 5-min extracellular applications of 1 mM $CaCl_2$ and 1 mM KCl (black strips at top; $n = 3$ experiments, 48, 24 and 32 stomata for wild type, *slac1-1* and *slac1-3*, respectively). Imposed intracellular Ca^{2+} transients (see Supplementary Fig. 7) were followed by 5-min exposures to a depolarizing solution containing 0 mM $CaCl_2$ and 50 mM KCl (white strips at top) as previously described²⁵. Stomatal apertures at time = 0 (100%) corresponded to average stomatal apertures of 3.56 ± 0.10 μ m, 3.93 ± 0.14 μ m and 3.56 ± 0.10 μ m in wild type, *slac1-1* and *slac1-3*, respectively. Error bars depict means $\pm$ s.e.m.

Figure 4. Ca^{2+} and ABA activations of S-type anion channels are impaired in *slac1* guard cells
a–d, Ca^{2+} activation of S-type anion channels. **a–c,** Whole-cell recordings of S-type anion currents in wild type (**a**), *slac1-1* (**b**) and *slac1-3* (**c**). **d,** Average current–voltage curves of S-type anion channel currents recorded in wild type (n = 7), *slac1-1* (n = 12) and *slac1-3* (n = 10). **e, f,** Typical R-type anion channel recordings (**e**), and average current–voltage curves in wild type (n = 3) and *slac1-3* (n = 6) (**f**). **g–j,** ABA activation of S-type anion channels. **g–i,** Typical recordings in wild type (**g**), *slac1-1* (**h**) and *slac1-3* (**i**). **j,** Average current–voltage curves recorded in wild type (n = 10), *slac1-1* (n = 8) and *slac1-3* (n = 8). Error bars depict means ± s.e.m.