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1 **A tandem amino acid residue motif in guard cell SLAC1 anion channel of grasses allows**
2 **for the control of stomatal aperture by nitrate**

3

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22 **Abstract**

23 The latest major group of plants to evolve were the grasses. These became important in the mid-
24 Paleogene about 40 million years ago. During evolution leaf CO_2 uptake and transpirational
25 water loss were optimized by the acquisition of grass specific stomatal complexes. In contrast
26 to the kidney-shaped guard cells (GCs) typical of the dicots such as Arabidopsis, in the grasses
27 and agronomically important cereals, the guard cells are dumbbell-shaped and are associated
28 with morphologically distinct subsidiary cells (SCs). We studied the molecular basis of guard
29 cell action in the major cereal crop barley. Upon feeding ABA to xylem sap of an intact barley
30 leaf, stomata closed in a nitrate dependent manner. This process was initiated by activation of
31 guard cell SLAC-type anion channel currents. HvSLAC1 expressed in Xenopus oocytes gave
32 rise to S-type anion currents that increased several fold upon stimulation with >3 mM nitrate.
33 We identified a tandem amino acid residue motif that within the SLAC1 channels differs
34 fundamentally between monocots and dicots. When the motif of nitrate-insensitive dicot
35 Arabidopsis SLAC1 was replaced by the monocot signature, AtSLAC1 converted into a grass-
36 type like nitrate-sensitive channel. Our work reveals a fundamental difference between monocot
37 and dicot guard cells and prompts questions into the selective pressures during evolution that
38 resulted in fundamental changes in the regulation of SLAC1 function.

39 **Introduction**

40 Guard cell pairs that drive stomatal movement control leaf CO_2 uptake and concomitant
41 transpirational water loss. To survive episodes of drought and excessive heat, stomata have to
42 sense sudden changes in environmental conditions and adjust stomatal aperture accordingly.
43 Based largely on work in the model dicot Arabidopsis much is known about the molecular
44 details that underlie stomatal function [1-3]. By way of contrast, in the grasses that are by far
45 the world's most important sources of food, it is surprising that our molecular knowledge
46 concerning guard cell function is still very limited [4-8] (and references therein).

47 In contrast to the kidney-shaped guard cells typical of the dicots, grass stomata come as pairs
48 of dumbbell-shaped guard cells (GCs) in physical contact with a lateral pair of subsidiary cells
49 (SCs, [4]). In terms of function there Raschke and Fellows (1971, [9]) found that during
50 stomatal opening in maize K^+ and anions are shuttled from SCs to GCs and when closing they
51 move in the opposite direction. Guard cells of dicots and monocots respond to the plant
52 hormone ABA, that binds to its cytosolic PYR/PYL/RCAR type receptor [10, 11]. The ABA
53 receptor forms a complex with the PP2C phosphatase ABI1 and SnRK2 kinase OST1 [12].
54 Binding of ABA to the receptor ABA cause inactivation of ABI1, and release of OST1 from
55 inhibition of the PP2C phosphatase [13]. In turn, the OST1 kinase phosphorylates the guard cell
56 anion channel SLAC1, causing it to open [14, 15]. The resulting release of anions depolarizes
57 the plasma membrane and the change in voltage activates the GORK1 channel resulting in K^+
58 efflux, which is followed by the loss of water and resulting stomatal closure [1, 16].

59 Potassium and chloride represent the dominant ions in dicot and monocot guard cells. In the
60 dicot model plant Arabidopsis, the SLAC1 channel is permeable to chloride. When expressed
61 in the heterologous expression system of *Xenopus* oocytes, Arabidopsis SLAC1 is active in
62 chloride-based media [14]. In contrast to SLAC1, its homologs SLAH2 and 3 require nitrate at
63 the external mouth of the anion channel to gate open in oocyte and guard cell systems [17, 18].
64 In order to gain insights into how cereal guard cells function, we analysed the transcript profile
65 of barley guard cells and subsidiary cells. We identified HvSLAC1 together with the major
66 components of guard cell ABA signalling. Strikingly, in barley SLAC1 and in marked contrast
67 to Arabidopsis SLAC1, we identified a distinct tandem amino acid motif, responsible for nitrate
68 activation.

69 **<u>Results</u>**

70 **ABA-dependent stomatal closure in barley requires extracellular nitrate**

71 Surprisingly, studies aimed at understanding the molecular basis of stomatal movements in
72 barley, a major cereal grass crop, are somewhat limited [4, 5]. To address this issue, we first
73 investigated stomatal function in the intact barley leaf using IRGA-based measurements of
74 stomatal conductance. We focussed on *Hordeum vulgare* line Barke, because genome
75 information is available, and it represents one of the most popular brewing barley varieties
76 worldwide.

77 From work in Arabidopsis it is known that ABA triggers stomatal closure following binding to
78 cytosolic receptors of the PYR/PYL-family and the subsequent induction of a signalling
79 pathway that ultimately activates SLAC/SLAH anion channels [14, 15, 17, 19, 20]. To
80 investigate the response of barley stomata to ABA, we fed 25µM ABA (a concentration
81 sufficient to close barley stomata [5]) via the leaf petiole. When the petiole was supplied with
82 ABA in water stomatal closure was delayed and incomplete (figure 1A). This is in marked
83 contrast to the prompt closure observed in the dicot Arabidopsis [21], but is similar to the
84 response observed in the monocot date palm [21]. However, the most striking result to emerge
85 from this experiment was that timely and significant ABA-induced stomatal closure could be
86 elicited by the addition of nitrate (5 mM KNO_3^-) to the feeding medium (figure 1B). This results
87 clearly shows that in barley the full stomatal response to ABA requires the presence of nitrate.

88

89 **Stomatal closure in barley involves inverse fluxes of K^+ and Cl^- between guard and**
90 **subsidiary cells**

91 Having identified a requirement for nitrate in ABA-induced closure we next investigated the
92 requirement for K^+ and Cl^-. Given that early studies in maize provided initial evidence for K^+
93 and chloride shuttling between guard cells and subsidiary cells [9, 22], we used EDX analysis
94 to determine the content of these elements in guard and subsidiary cells. Figure 1C shows that
95 guard cells (GC) from closed stomata contained less K and Cl than open ones. Subsidiary cells
96 (SC), however, exhibited the inverse relationship with relatively higher levels of K and Cl
97 associated with closed stomata (figure 1C). These data confirm older work in maize [9, 22] but
98 leave open the question of why there is a requirement for nitrate for the closure of barley
99 stomata. In summary, in barley just as in Arabidopsis, K^+ and Cl^- represent major ionic
100 components of the guard cell osmotic motor that drives stomatal movement. The fact that the

101 changes in K^+ are larger than those of Cl^-, indicate that besides Cl^-, additional anions such as
102 nitrate or malate must be involved. In this context, it should be mentioned that in barley
103 epidermal cells Cl^- and NO_3^- might provide the total negative solute charge [23].

104

105 **Using transcriptomics to investigate ABA-induced stomatal closure in barley**

106 To investigate the molecular machinery responsible for bringing about stomatal closure in
107 barley and in particular the ion fluxes between guard and subsidiary cells, we employed a
108 transcriptomic approach. We isolated three experimental preparations; 1) intact leaves (L); 2)
109 lower epidermis with intact stomatal complexes (GCSC) and 3) leaves without upper and lower
110 epidermis und thus without guard cell complexes (LwoGC). We employed a bioinformatic
111 approach to identify differentially expressed genes (DEG) in guard cell complexes (for full
112 DEG list see table S1). Because guard cells, in contrast to mesophyll cells, contain a reduced
113 number of chloroplasts and epidermal pavement cells have no chloroplasts, we initially
114 compared photosynthesis (PS) related transcripts. As expected, we found genes associated with
115 PS electron transport and CO_2 fixation underrepresented in the GCSC samples (table S1, filtered
116 by MapMan category 1). When analysing RNA-seq data, it did not come as a surprise that we
117 found DEGs related to guard cell function- and ABA signalling.

118 *We next investigated genes involved in ion transport.* In the ion channel fraction guard cell-
119 specific anion channels of the *SLAC/SLAH* type and Shaker-like potassium channels of the
120 *KAT*- and *GORK/SKOR*-type were identified (table S2). This expression pattern underpins the
121 notion that the grass type stomata from barley harbour the guard cell major H^+, K^+ and Cl^-
122 transporting entities represented by *AHA1* and *KAT1* for stomatal opening [24] and *SLAC1* and
123 *GORK* for closure ([1] for review). Among the ABA dependent transcripts, pronounced guard
124 cell expression of orthologues to the *PYR/PYL* ABA receptor family, *PP2C* phosphatases *ABI1*
125 and *2*, *SnRK2* kinases of the *OST1*-type were found. Based on 5 *HvOST1*-like sequences, we
126 generated a phylogenetic tree and identified *HvOST1.1, 1.2* and *1.3* most closely related to the
127 Arabidopsis *OST1* (figure S1A).

128 To validate expression of the guard cell ion channels and components of the ABA signalling
129 pathway suggested by the RNA-seq data, we performed qPCR analysis. We sampled leaves (L)
130 and epidermal peels containing both guard cells and subsidiary cells (GCSC) and preparations
131 in which the subsidiary cells were selectively disrupted using the blender method [25]. The
132 latter preparation represented a fraction highly enriched in guard cells (GC) (figure S1B). As in
133 Arabidopsis guard cell databases obtained using a related experimental approach [25], we found

134 transcripts of *ABI1*, *SLAC1* and *OST1.1* expressed in an almost guard cell specific manner. In
135 contrast, *OST1.3* expression was found distributed equally among samples. Among the guard
136 cell expressed *SnRK2* genes, HvOST1.1 shows the closest phylogenetic relationship to the
137 Arabidopsis OST1 kinase with 80.5% identical residues (figure S1A). The HvOST1.1 sequence
138 harbours all known functional domains of OST1-like, ABA-dependent SnRK2 kinases,
139 including the DI domain/SnRK2 box and the DII domain/ABA-Box [12, 26-28]. On these
140 grounds HvOST1.1 very likely represents the SLAC1 activating ABA kinase in barley. In
141 Arabidopsis GCs SLAH3 operates a nitrate activated anion channel conducting nitrate and
142 chloride [17]. In barley, however, *SLAH3.1* was found expressed in leaves, but not in the GCSC
143 and GC samples (figure S1B).

144

145 **Barley HvSLAC1 is under the control of nitrate and OST1 /ABI1 pair**

146 McAdam et al., 2016 [29] and Lind et al., 2015 [30] showed that SnRK2 kinases (OST1s) are
147 strictly conserved during evolution. All OST1 kinases derived from different evolutionary
148 distinct plant species so far tested are capable of activating Arabidopsis SLAC1. In addition,
149 AtOST1 is capable of activating SLAC1 isoforms from other monocot species, such as
150 PdSLAC1 from date palm [21] or OsSLAC1 from rice [8]. Thus, to understand the molecular
151 basis of nitrate dependency of stomatal closure in barley, we expressed HvSLAC1 alone and
152 together with AtOST1 in Xenopus oocytes and studied its anion channel properties. In the
153 absence of the SnRK2 kinase no currents were recorded and even in the presence of the ABA-
154 induced kinase AtOST1 and 30 mM chloride-based extracellular media, macroscopic S-type
155 anion currents could only be recorded at strongly depolarized membrane potentials (figure 2A).
156 Upon addition of 30 mM nitrate, however, pronounced S-type anion currents were observed
157 (figure 2A). While the Arabidopsis OST1 WT kinase was capable of activating HvSLAC1 in
158 nitrate-based solutions, the kinase dead mutant AtOST1 D140A could not perform this function
159 (figure 2B and C). This indicates that phosphorylation of HvSLAC1 is strictly required for
160 anion channel activation (cf. [14]). Besides the calcium independent SLAC1 kinase OST1,
161 calcium dependent kinases of the CPK and CIPK/CBL type can phosphorylate and gate open
162 AtSLAC1 ([31]). As a representative of the latter kinases category, we selected CPK6 for
163 oocyte co-expression experiments with HvSLAC1. As with OST1, CKP6 activated the barely
164 S-type channel to the same extent as AtSLAC1 (figure 2A; figure S2A; [19, 32]).

165 In contrast to the grass SLAC1, Arabidopsis SLAC1 does not require the presence of
166 extracellular nitrate for activation (figure S2A, c.f. [18]). Interestingly, the ABA phosphatase

167 ABI1 in *Arabidopsis* guard cell inhibits the response to nitrate [33] and negatively regulates
168 AtOST1 and AtSLAC1 activity [14, 15, 19, 31]. Upon co-expression of HvSLAC1 and AtOST1
169 with ABI1, we found that HvSLAC1-mediated anion currents were strongly reduced (figure 2B
170 and C). These findings indicate that fast ABA signalling is conserved between guard cells of
171 the dicot *Arabidopsis* and monocot grass *Hordeum vulgare*.

172 The Arabidopsis AtSLAC1 does not require extracellular nitrate for activation (figure S2A) but
173 has a strong permeably preference for nitrate over chloride [14]. The HvSLAC1 P_{NO3}/P_{Cl}
174 calculated permeability ratio of 6.4 ± 0.8 indicates that the dicot and monocot guard cell anion
175 channel would preferentially conduct nitrate when present in guard cells at osmotically relevant
176 quantities (figure S2B). Is the nitrate sensitivity a unique feature of HvSLAC1 or is it found in
177 other cereals, too? To answer this question we expressed rice SLAC1 (OsSLAC1) in oocytes
178 (cf. [8]). The results in figure S2A and C show that the rice S-type anion channel shared its
179 selectivity and nitrate dependent features with the Hordeum SLAC1 anion channel. In this
180 context, it should be noted that we recently showed, in the monocot *Phoenix dactylifera* (date
181 palm), that PdSLAC1 is also nitrate activated [21]. Besides nitrate, other physiological relevant
182 anions such as phosphate, sulphate, malate or chloride were not capable of activating
183 HvSLAC1-derived anion currents (figure S2D) or to shift its rel. P_O to negative (physiological)
184 membrane potentials (figure S2E).

185 To find out whether nitrate activation is a property of monocot SLAC1, we compared the
186 Brassicaceaen AtSLAC1 with a dicot orthologue from tomato and tobacco - two Solanaceae
187 crop species. These dicot SLAC1s behave more similarly to the nitrate insensitive AtSLAC1
188 than to nitrate-activated monocot SLAC1s (figure 2D).

189

190 **Extracellular nitrate primes HvSLAC1 to release chloride**

191 To study the biophysical properties of nitrate-activated HvSLAC1 in more detail we co-
192 expressed the anion channel with AtCPK6 [2, 19, 32] and determined current densities and the
193 relative open probability as a function of the external nitrate concentration (figure 3A and B).
194 When exposed to increasing external nitrate concentrations, the peak efflux currents and the
195 relative open probability shifted towards negative membrane potentials and thereby increased
196 the plasma membrane anion conductance (figure 3A and B). In contrast, similar experiments
197 with increasing external chloride applications revealed that in the physiological membrane
198 potential range (negative from -100 mV), anion release currents are absent irrespective of the
199 external Cl^- concentration (figure 3C and D). While 100 mM nitrate shifted the HvSLAC1 half-

200 maximal open probability ($V_{1/2}$) to -120 mV (figure 3B), $V_{1/2}$ in 100 mM chloride remained at
201 depolarized membrane potentials of -20 mV (figure 3D). When plotting $V_{1/2}$ as a function of
202 the external nitrate concentration, the resulting saturation curve could be described with a
203 Michaelis-Menten equation (figure 3E) resulting in a $K_{0.5}$ value of 10.9 ± 3.8 mM nitrate. Thus
204 physiological [NO_3^-] concentration of 10 to 70 mM found in the xylem sap of barley leaves
205 [34, 35] will activate HvSLAC1 anion channel. This set of experiments shows not only that
206 HvSLAC1 conducts nitrate (figure 3A and B, figure S2B) but also that nitrate is required to
207 gate the barley guard cell channel open.

208 Nitrate-dependent gating is a known feature of SLAH2 and SLAH3 branch of the Arabidopsis
209 SLAC/SLAH anion channel family, but not of its founding member AtSLAC1 (see above and
210 c.f. [17, 18]; figure S2A). While AtSLAH2 is strictly nitrate selective, AtSLAH3 also conducts
211 chloride when primed with extracellular nitrate [17, 18]. To further examine the nature of the
212 nitrate dependency of HvSLAC1 and to substantiate that HvSLAC1 conducts chloride in the
213 presence of its gating ligand nitrate, the anion channel was challenged by different chloride to
214 nitrate ratios. Anion currents recorded in the presence of 3 mM extracellular chloride were very
215 weak and reversed at +50 mV (figure 3F). In contrast, addition of 3 mM nitrate enhanced the
216 steady state currents and shifted the reversal potential to 0 mV (figure 3F). When the chloride
217 concentration was further increased to 100 mM in the presence of 3 mM nitrate, the reversal
218 potential of HvSLAC1-mediated anion currents shifted to more negative membrane potentials
219 without anion release currents and relative open probability being influenced by chloride (figure
220 3F and G). To further investigate the chloride conductance of HvSLAC1 when primed with
221 extracellular nitrate, we monitored the reversal potential of HvSLAC1 AtCPK6 expressing
222 oocytes. Upon addition of 3 mM nitrate to a 3 mM chloride containing bath solution, the
223 reversal potential dropped by 56 mV and shifted to even more negative membrane potentials
224 when the chloride concentration was increased to 100 mM (figure S2F and G). This behaviour
225 and those to varying Cl^- to NO_3^- rations (figure 3F and G) indicate that HvSLAC1, when pre-
226 activated by nitrate, conducts both nitrate and chloride. In contrast to the nitrate sensitive
227 monocot S-type anion channels, AtSLAC1 reversal potential shifts appeared less nitrate- but
228 more chloride sensitive (figure S2F and G). In contrast to AtSLAC1, OsSLAC1 and HvSLAC1,
229 the reversal potential of the nitrate-selective AtSLAH2 was sensitive to nitrate only but not
230 chloride (figure S2F and G). Taken together, the electrical properties of the monocot anion
231 channels are reminiscent of the nitrate-gated, chloride and nitrate permeable AtSLAH3 anion
232 channel rather than of the nitrate-independent dicot AtSLAC1.

233

234 **SLAC1 grass type tandem amino acid motif on TMD3 is key for nitrate priming**

235 3D homology modelling of AtSLAC1 and AtSLAH2 to the crystal structure obtained with the
236 bacterial homologue HiTehA in combination with site-directed mutagenesis showed associated
237 residues of Trans-Membrane Domain TMD3 as part of the pore forming entity [18, 36]. To find
238 the nitrate site in barley SLAC1, we compared TMD3 of monocot and dicot SLAC1 type
239 channels (figure 4A, figure S3). Monocot and dicot SLAC1s could be well distinguished by
240 two residues close to Val272 and Val273 of AtSLAC1. We found dicot SLAC1s to either carry
241 two valine residues such as AtSLAC1 (V272 and V273) or an IV pair in Solanaceaen species.
242 Monocots including barley HvSLAC1 and date palm PdSLAC1 at the related positions harbour
243 an isoleucine and alanine side chain (figure 4A, Fig S3) similar to the nitrate activated SLAH2/3
244 anion channels that possess either the IA or an IS motif (figure S3). Given that the amino acid
245 sequence on TMD3 clearly distinguishes monocot from dicot SLACs, these residues seem to
246 represent a specific signature. Thus, we tested whether this TMD3 tandem motif between both
247 monocot and dicot representative SLACs is essential for nitrate dependency. Therefore, we
248 replaced just the VV motif in AtSLAC1 by IA and the IA motif in Hv/PdSLAC1 by the dicot
249 VV motif. The resulting Arabidopsis mutant AtSLAC1 V272I V273A displayed nitrate-
250 induced anion currents just like HvSLAC1 and PdSLAC1 WT (figure 4B). Note, this behaviour
251 appeared only in 30 % of the tested oocyte batches (this conditional phenotype is shown in
252 figure 4B) whereas the remaining oocyte batches revealed a AtSLAC1 WT behaviour. Thus,
253 the introduction of the monocot IA motif in TMD3 of AtSLAC1 is essential and sufficient to
254 provide for the nitrate dependency. However, when the monocot SLACs were equipped with
255 the dicot VV signature the resulting mutants with barley (HvSLAC1 I286V A287V) and date
256 palm anion channel (PdSLAC1 I285V A286V), appeared severely impaired even in nitrate-
257 based buffers (figure 4B) indicating that in monocots additional structural moieties shape the
258 permeation pathway. The fact that the HvSLAC1 I286V A287V and the PdSLAC1 I285V
259 A286V mutant did not carry macroscopic anion currents and that AtSLAC1 V272I V273A
260 displayed a conditional phenotype only, indicates that we have identified a critical position
261 within the selectivity filter in the anion channels' pore that might result in a meta-stable
262 structure in the mutant AtSLAC1 V272I V273A. Thus, it is tempting to speculate that for proper
263 anion discrimination additional residues are involved.

264 To find a molecular explanation to this discrepancy in monocot-dicot pore residue exchange,
265 we thus asked which channel sites co-evolved with the TMD3 signature. Using a bioinformatics

266 co-evolution pipeline (www.evfold.org), we identified residues in AtSLAC1 that co-evolved
267 with the signature motif. Interestingly, the 50 highest scoring hits of co-evolved residues were
268 found exclusively on TMD1 to 3 but not on other parts of the SLAC1 protein (figure 4C, Table
269 S3). To test whether TMD1 to 3 including the remarkable difference in TMD3 between
270 monocot and dicot SLAC1 representatives is essential and sufficient to provide for the monocot
271 nitrate dependency and dicot nitrate independency, we exchanged either only TMD1 and 2 or
272 TMD1, 2 and 3 between AtSLAC1 and HvSLAC1. The resulting chimeras were named
273 AtSLAC1(HvTMD1-2) where AtSLAC1 carries TMD1 and 2 from HvSLAC1,
274 AtSLAC1(HvTMD1-3) where AtSLAC1 carries TMD1-3 from HvSLAC1 and vice versa.
275 When comparing WT SLAC1 channels with the chimeras where only TMD1 and 2 was
276 replaced, we could not document any change in their chord conductance (figure 4D). Only when
277 TMDs 1-3 were exchanged, the nitrate-dependency of HvSLAC1 could be transferred to
278 AtSLAC1 while HvSLAC1 lost its nitrate-dependency when equipped with TMD1-3 of
279 AtSLAC1 (figure 4D). The comparison of rel. open probabilities between AtSLAC1 WT and
280 AtSLAC1(HvTMD1-3) demonstrates that the activation of the chimera is based on a nitrate-
281 dependent shift of its rel. P_O to negative membrane potentials just like with monocot HvSLAC1
282 WT (figure 4E and F). On the contrary, the chimera HvSLAC1(AtTMD1-3) appeared open
283 even in the absence of nitrate, just like dicot AtSLAC1 WT (figure 4E and F). Thus, the TMD3
284 IA signature from monocots is required and sufficient to convert the dicot SLAC1 from
285 Arabidopsis into a nitrate-gated monocot grass SLAC1 anion channel (figure 4B). In contrast,
286 monocot SLAC1s require the VV motif and in addition backbone residues situated on TMD1
287 and 2 to be converted in a nitrate-insensitive anion channel (figure 4D to F).

288 **Discussion**

289 One of the most striking results to emerge from the experiments described in this paper is that
290 the rates of stomatal opening and closure in barley are much faster than in Arabidopsis (cf.
291 [37]). In the absence of other compensatory factors this is likely to confer a selective advantage
292 on barely in comparison with Arabidopsis. The ability to rapidly adjust stomatal aperture to suit
293 the prevailing environmental conditions is likely to allow barley enhanced control over
294 transpiration, xylem-based nutrient delivery, and photosynthesis that will likely play out in
295 terms of increased competitiveness ([4, 38], and refs. therein).

296 In seeking a mechanistic explanation for the ability of barley stomata to open and close rapidly
297 we focussed first on the flux of the major osmotically active cations and anions. Our results
298 suggest that barley has evolved a stomatal system in which the guard and subsidiary cells
299 behave as a functional unit [4, 37]. For example, during closure while there is loss of K^+, Cl^-
300 (figure 1C) and very likely NO_3^- too from the guard cell, it accumulates in the subsidiary cells.
301 In this sense, the subsidiary cells have evolved to play a role as reservoirs or cisterns of
302 osmotically active ions. In grasses stomata move faster than those of dicots because guard cells
303 and subsidiary cells actively regulate turgor and volume in an inverse manner [4, 9]. This way
304 subsidiary cells operate as a source of osmotica that is used by guard cells when they swell, and
305 the stomatal pore opens (figure 1C).

306 A comparative transcriptomic approach of ABA signalling in the cells of the barley stomatal
307 complex revealed the presence of components that were well known from investigations of
308 Arabidopsis guard cells (table S2). These data suggested that, in addition to the evolution of
309 functionally linked and morphologically distinct guard and subsidiary cells, we should look for
310 augmentation of known players in addition to novel elements to explain the rapid movements
311 in barley stomata.

312

313 **A tandem amino acid signature in monocot SLAC1 anion channels provide for nitrate**
314 **dependent gating**

315 Here we focussed on the barley SLAC1 anion channel. The regulation of this channel by ABI1
316 and OST1 appeared to be highly conserved between Arabidopsis and barley (figure 2B and C,
317 [14, 15]). The most striking feature to emerge was that gating of the barley guard cell anion
318 channel is controlled by ABA signalling and nitrate (figure 2 and 3). Using a structural biology
319 approach coupled with site-directed mutagenesis, we identified the key residues located in

320 TMD3 that are responsible for nitrate gating (figure 4). Interestingly, structure-function
321 investigations with AtSLAH2 also identified Serine 228 (equivalent to V273 in AtSLAC1) in
322 TMD3 as the key residue for the strict nitrate selectivity of the root anion channel [18]. Monocot
323 SLACs could only be converted to nitrate independent dicot-like anion channels when TMD3
324 together with TMD1 and 2 were exchanged (figure 4D to F). This indicates that during SLAC1
325 evolution in monocots, residues on TMD1 and 2 coevolved together with the IA motif in TMD3
326 to form the nitrate gating site (figure 4C, Table S3). Whether and how the intrinsic nitrate sensor
327 in monocot SLAC1 anion channels contribute to the evolutionary success of cereals remain to
328 be shown. However, it is tempting to speculate that SLAC1 anion channels harbouring an
329 intrinsic nitrate sensor might allow the plant to integrate leaf nitrate levels and the
330 velocity/degree of stomatal closure.

331

332 **Evolution of the SLAC1 pore properties**

333 We and others showed that SLAC1 channels in dicots are permeable to chloride and nitrate and
334 do not require nitrate as gating modifier (this study and [14, 15, 17, 39]). In contrast, monocot
335 SLAC1 channels, such as HvSLAC1, PdSLAC1 and OsSLAC1, require nitrate at the
336 extracellular face of the anion channel pore to gate open, which is reminiscent of the nitrate
337 dependent gating of AtSLAH3 and PttSLAH3 anion channels [17, 31, 40, 41]. Sequence
338 comparisons identified a VV signature in dicot and an IA signature in monocot SLACs that
339 clearly differ between these two evolutionary distinct plant lineages (figure S3 and S4A).
340 Interestingly, similar to monocot SLAC1s, nitrate activated SLAH2/3 anion channels also
341 possess either an IA or an IS motif on TMD3 (figure S3).

342 SLAC1-type anion channels are found in the most basal land plants such as green algae
343 *Klebsormidium nitens* as well as in the liverwort *Marchantia polymorpha* [30]. With the
344 emergence of stomata in mosses such as *Physcomitrella patens,* SLAC1 anion channels co-
345 opted the fast ABA-signaling cascade and became OST1-sensitive [30]. figure S3 and S4A
346 shows that the IA motif appeared in moss first and remained largely conserved until the
347 emergence of Arecales (date palm) and Poales lineages that include important grass crops such
348 as rice, maize and barley. In the latter monocots, nitrate-dependent gating is fully functional
349 (this study and [21]). This raises the question of when in evolution SLAC1-type anion channels
350 carrying an IA motive evolved nitrate-dependent gating?

351 To answer this question, we analysed the chord conductance of a set of SLAC1 anion channels
352 derived from evolutionary distinct basal plant lineages including the moss *Physcomitrella*

353 *patens*, the lycophyte *Selaginella moellendorffii*, the fern *Ceratopteris richardii* and the
354 seagrass *Zostera marina*. Apart from the moss PpSLAC1, all tested basal SLAC1 anion
355 channels appeared equally nitrate insensitive as the dicot SLAC1 anion channels from
356 Arabidopsis, tobacco and tomato (figure S4B, figure 2D). This may indicate that nitrate
357 dependent gating evolved as recently as the emergence of monocot species, although the IA
358 motif on TMD3 is already established in a majority of SLAC-type anion channels of basal plant
359 lineages (figure S3 and S4A). In contrast, following the split between dicots and monocots, the
360 SLAC1s from dicot species lost the IA signature and did not develop a nitrate dependent gating
361 mechanism (figure S4A).

362 To further support that nitrate dependent gating in monocot species evolved after the split
363 between monocots and dicots, we employed a probabilistic approach [42], to infer the most
364 probable core SLAC1 sequence (TMD1 to 10) of the common ancestor from which all extant
365 dicot and monocot SLAC1s evolved (figure S4A). This inferred core sequence was synthesized,
366 equipped with the N- and C-terminus of AtSLAC1 and named Ancestral Slow Anion Channel
367 1 (AncSLAC1, sequence can be found in table S4). Interestingly, AncSLAC1 carried the IA
368 motif on TMD3 just like monocot and basal SLAC1s. Following co-expression with CPK6 in
369 *Xenopus leavis* oocytes, AncSLAC1 displayed typical S-type anion currents that slowly
370 deactivated at hyperpolarized membrane potentials (figure S4C). In line with SLAC1s from
371 dicots but in contrast to nitrate activated monocot SLAC1 channels, AncSLAC1 mediated
372 macroscopic anion currents in both chloride and nitrate-based media (figure S4B and C) and
373 showed no nitrate dependent gating behavior (figure S4D). Thus, both the predicted common
374 ancestor of dicot and monocot SLAC1 channels AncSLAC1 as well as SLAC1 channels from
375 basal plant lineages were equipped with the IA motif on TMD3 but displayed a largely nitrate
376 independent gating behavior, suggesting that monocots have evolved the nitrate dependent
377 gating mechanism after the split from the dicot species. Future studies will address the question,
378 which backbone sites had to emerge in TMD1 and/or 2 together with the IA motif to form a
379 nitrate-sensitive SLAC1 gate in monocots.

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386

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389 T.M., M.E.J., P.A., J.F., D.G., A.M.H., and R.H. wrote the manuscript.

390

391 **Declaration of interests**

392 The authors declare that there is no competing financial interest.

393

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584

585 **Figure legends**

586 **Figure 1: ABA induced stomatal closure requires NO_3^-.** Barley stomatal movement
587 measured by infrared gas exchange. Barley stomata were opened in the light (400 µE) at
588 ambient CO_2 (400 ppm). (A) Excised leaves were supplied with water and ABA via the
589 transpiration stream. Note, stomatal closure in the presence of ABA was remarkably slow and
590 largely incomplete. (B) Leaves were pre-incubated with 5 mM nitrate. Under this condition
591 leaves started to close their stomata 5 min after ABA application. This process was largely
592 completed within 10 minutes and finished in less than 15 min. Data were normalized to their
593 open value 10 minutes before the application of ABA (indicated by the arrow). $n \geq 5$ means ±
594 SE. (C) EDX-analysis of barley stomatal complexes. The respective changes in K- (left) and
595 Cl-contents (right) are shown after the transition from open to closed stomata in guard cells
596 (GC) and subsidiary cells (SC). During stomatal opening potassium and chloride ions shuttle
597 from subsidiary cells to guard cells, while ions move in the opposite direction when stomata
598 close. n = 11, means ± SE.

599 **Figure 2. Nitrate-dependent activation of HvSLAC1. See also figure S2.** (A) Whole-oocyte
600 currents of *Xenopus* oocytes expressing HvSLAC1 alone or co-expressing either AtOST1 or
601 AtCPK6 were measured in response to the standard voltage protocol. Currents were recorded
602 in standard buffers containing either 3 mM chloride, 30 mM chloride or 30 mM nitrate.
603 Representative cells of 2 independent experiments with n = 3 oocytes are shown. **See also**
604 **figure S2A.** (B) Whole-oocyte currents of oocytes expressing HvSLAC1 equipped with the C-
605 terminal half of YFP (HvSLAC1:YC) either expressed alone or together with WT OST1, OST1
606 D140A or WT OST1 and ABI1. Both OST1 versions were fused to the N-terminal half of YFP
607 (OST1:YN, OST1 D140A:YN). Currents were recorded in nitrate-based buffers (30 mM).
608 Representative cells are shown. Co-expression of HvSLAC1 and OST1 or OST1 D140A was
609 confirmed by detection of YFP fluorescence. Quarter of representative oocytes of 2 independent
610 experiments with n = 4 oocytes are shown. (C) Statistical analysis of the steady-state currents
611 at -100 mV derived from the experiment described in (B) (n = 4 experiments, mean ± SD). (D)
612 Chord conductance recorded at a membrane potential of -120 mV of oocytes co-expressing
613 AtOST1 with SLAC1 from different plant species indicated in the figure. Chord conductance
614 was calculated from instantaneous currents recorded in chloride- or nitrate-based buffers (100
615 mM). Chord conductance in nitrate was set to 1 ($n \geq 4$ from 2 independent experiments, mean
616 ± SD).

617 **Figure 3: Nitrate activates HvSLAC1 by shifting its rel. open probability to**
618 **hyperpolarized voltages. See also figure S2.** (A) Nitrate-dependence of steady-state currents
619 (I_{SS}) of oocytes co-expressing HvSLAC1 and AtCPK6 are plotted as a function of the applied
620 membrane potential (n = 4 from 2 independent experiments, mean ± SD). (B) The relative open
621 probability (rel. P_O) measured in different NO_3^- concentrations of HvSLAC1/AtCPK6-
622 expressing oocytes was plotted against the membrane potential. Data points were fitted with a
623 single Boltzmann equation (solid lines, n = 4 from 2 independent experiments, mean ± SD).
624 (C) Steady-state currents (I_{SS}) of oocytes co-expressing HvSLAC1 and AtCPK6 recorded in the
625 presence of different external chloride concentrations or 30 mM nitrate (n = 4from 2
626 independent experiments, mean ± SD). (D) The relative open probability (rel. P_O) of HvSLAC1
627 in the presence of different Cl^--concentrations or 30 mM NO_3^- was plotted against the
628 membrane potential. Data points were fitted with a single Boltzmann equation (solid lines, n =
629 4 from 2 independent experiments, mean ± SD). (E) The half-maximal P_O ($V_{1/2}$) calculated from
630 the data in (B) was plotted against the nitrate concentration. A Michaelis-Menten equation was
631 used to calculate a $K_{0.5}$ of 10.9 mM NO_3^- (n = 4, mean ± SD). (F) Steady-state currents of
632 HvSLAC1 and AtCPK6 co-expressing oocytes in the presence of different Cl^-/NO_3^--ratios were
633 plotted against the applied voltage (n = 4 from 2 independent experiments, mean ± SD). (G)
634 The relative open probability (rel. P_O) of HvSLAC1 in different Cl^-/NO_3^--ratios was plotted
635 against the membrane potential. Data points were fitted with a single Boltzmann equation (solid
636 lines, n = 4 experiments, mean ± SD).

637 **Figure 4: IA-motif on TMD3 coevolved with residues on TMD1 and 2 to provide monocot**
638 **SLAC1 anion channels with a nitrate-depending gating mechanism. See also figure S3 and**
639 **S4 as well as table S3 and S4.** (A) Frequency loges of transmembrane three (TMD3) from
640 SLAC1 anion channels of different monocot or dicot species. The respective sequence
641 alignment is shown in figure S3. The most prominent difference (IA motif in monocots vs.
642 VV/IV motif in dicots) is marked with a red box. (B) Chord conductance at -120 mV of
643 AtSLAC1 WT and AtSLAC1 V272I V273A compared to HvSLAC1 WT and HvSLAC1 I286V
644 A287V or PdSLAC1 WT and PdSLAC1 I285V A286V. All channels and mutants thereof were
645 co-expressed with CPK6. Currents were recorded in nitrate- or chloride-based buffers. (n = 4
646 from 2 independent experiments, mean ± SD). Note, the phenotypes of HvSLAC1 and
647 AtSLAC1 anion channels were highly reproducible showing nitrate-independent gating
648 properties of AtSLAC1 and nitrate-dependent gating of HvSLAC1 expressing oocytes. In
649 contrast, with the mutant AtSLAC1 V272I V273A we observed a conditional phenotype
650 strongly dependent on the investigated oocyte batch. In 30 % of the tested oocyte batches the

651 mutations in the selectivity signature converted AtSLAC1 into a HvSLAC1-type nitrate-
652 dependent anion channel (these data are shown in this study) whereas the remaining oocyte
653 batches revealed a AtSLAC1 WT behaviour. (C) Evolutionary coupling analysis. The top 50
654 amino acid residues (**see also table S3**) that showed evolutionary coupling to AtSLAC1-V272
655 and V273 (purple spheres) were highlighted in red on previously generated homology models
656 [18, 36] using VMD [43]. Note, some of the highlighted residues co-evolved with both V272
657 and V273. The sphere size of co-evolved residues does not relate to the evolutionary coupling
658 strength but reflects the side chain size. TMD1 is depicted in dark grey, TMD2 in green and
659 TMD3 in light grey. The remaining TMDs are shown in transparent orange. (D) Chord
660 conductance of oocytes co-expressing AtCPK6 with either AtSLAC1, HvSLAC1 or one of the
661 indicated chimeras. Currents were recorded in nitrate or chloride-based buffers. Chord
662 conductance for nitrate was set to 1 (n = 4 from 2 independent experiments, mean ± SD). **See**
663 **also Figure S4B.** (E) and (F) Relative open probability (rel. P_O) of (E) AtSLAC1 and the
664 chimera AtSLAC1(HvTMD1-3) or (F) HvSLAC1 and HvSLAC1(AtTMD1-3) in the presence
665 of 30 mM chloride or nitrate (n=4 from 2 independent experiments, mean ± SD).

666

667 **STAR Methods**

668 **CONTACT FOR REAGENT AND RESOURCE SHARING**

669 Further information and requests for resources and reagents should be directed to and will be
670 fulfilled by the Lead Contact, Dietmar Geiger (geiger@botanik.uni-wuerzburg.de).

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671

672 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

673 **Plant material and growth conditions**

674 Barley (*Hordeum vulgare* cv. Barke) seeds were provided by a commercial supplier (Saatzucht
675 J. Breun GmbH & Co. KG) and cultivated at 22/16 °C and 50 ± 5% RH at a 12/12h day/night
676 cycle and a photon flux density of 500 µmol m-2 sec-1 white light (Philips Master T Green
677 Powers, 400 W).

678 **Xenopus oocyte preparation**

679 Investigations on SLAC1 anion channels were performed in oocytes of the African clawfrog
680 *Xenopus laevis*. Permission for keeping Xenopus exists at the Julius-von-Sachs Institute and is
681 registered at the government of Lower Franconia (reference number 70/14). Mature female
682 *Xenopus laevis* frogs (healthy, non-immunized and not involved in any previous procedures)
683 were kept at 20 °C at a 12/12h day/night cycle in dark grey 96 litres tanks (5 frogs/tank). Frogs
684 were fed twice a week with floating trout food (Fisch-Fit Mast 45/7 2mm, Interquell GmbH,
685 Wehringen, Germany). Tanks are equipped with 30 cm long PVC pipes with a diameter of
686 around 10 cm. These pipes are used as hiding places for the frogs. The water is continuously
687 circulated and filtered by a small aquarium pump. For oocyte isolation, mature female *X. laevis*
688 frogs were anesthetized by immersion in water containing 0.1% 3-aminobenzoic acid ethyl
689 ester. Following partial ovariectomy, stage V or VI oocytes were treated with 0.14 mg/ml
690 collagenase I in Ca^{2+}-free ND96 buffer (10 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1
691 mM $MgCl_2$,) for 1.5 h. Subsequently, oocytes were washed with Ca^{2+}-free ND96 buffer and
692 kept at 16 °C in ND96 solution (10 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_{2,}$
693 1mM $CaCl_2$) containing 50mg/l gentamycin. For oocyte BiFC and electrophysiological
694 experiments 10 ng of each cRNA was injected into selected oocytes. Oocytes were incubated
695 for 2 days at 16 °C in ND96 solution containing gentamycin.

696

697 **METHODS DETAILS**

698 **RNA sequencing**

699 Epidermal peels were collected from the abaxial side of 8 to 10-day-old leaves. To prepare
700 isolated epidermal peels [5], leaves were cut from the plant and bent over the forefinger with
701 the adaxial surface facing upward. A shallow cut was made with a sharp razor blade horizontally
702 across the leaf and a flap of leaf tissue lifted with a razor, leaving the lower epidermis intact.
703 The leaf tissue was removed from the epidermis with forceps. RNA was extracted from a total
704 of 20 epidermal peels per sample using the NucleoSpin® RNA Plant Kit (Macherey-
705 Nagel,Drueren, Germany). RNA isolation from whole leaves was performed similarly.

706 The extracted RNA was treated with RNase-free DNase (New England Biolabs, Ipswich, MA,
707 USA). Quality control measurements were performed on a 2100 Bioanalyzer (Agilent, Santa
708 Clara, CA, USA) and the concentration was determined using a Nanodrop ND-1000
709 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Following RNA
710 isolation, we sequenced RNA of one sample, each to get a first overview of genes in barley
711 guard cell complexes that are known to be involved in stomatal movements. In addition, we
712 were thus able to obtain the sequence information for cloning selected transporters and
713 channels. Libraries were prepared with the TruSeq RNA Sample Prep Kit v2 (Illumina, San
714 Diego, CA, USA) using 1 µg of RNA and sequenced on a HiSeq 3000 (Illumina) resulting in a
715 sequence depth of 35 million paired-end reads (2x 150bp).

716 **RNA-seq data analysis**

717 Sequencing adaptors were initially removed, and the overall high quality of the remaining reads
718 was confirmed using FastQC (FASTQC v0.10.1, Andrews:
719 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (average Phred quality score of
720 >30 across all bases at each position in the FastQ files). Subsequently, reads were aligned to
721 the barley reference assembly [44] using Hisat2 (hisat2-2.0.3-beta) [45] by applying default
722 settings for paired-end data. The featureCounts function of the subread-1.4.6 package [46] was
723 used to generate counts for high-confidence genes of barley [44]. Only uniquely mapped read
724 pairs were counted. Data normalization was performed by calculating TPM (Transcripts Per
725 Kilobase Million) [47] values.

726 **Functional annotation of barley**

727 The functional annotation of the barley genes was provided by Mascher et al. (2017 [44]). Best
728 hits of a BLASTP [48] alignment of barley high-confidence protein sequences against the *A.*

729 *thaliana* protein sequences (TAIR10, [49]) were used to assign *A. thaliana* genes and the
730 corresponding MapMan categories [50] to barley genes.

731 **Gas exchange experiments**

732 For gas exchange measurements [21], we used detached leaves of 8 to 10-day-old *Hordeum*
733 *vulgare* cv. Barke. The leaves were cut under water to avoid xylem embolism and immediately
734 placed in deionized water or 5 mM KNO_3 and kept there for the whole measurement period.
735 The effect of ABA application with or without KNO_3 on the transpirational water loss was
736 measured at a photon flux density of 500 µmol $m^{-2}s^{-1}$. After stabilization of the transpiration
737 ABA with a final concentration of 25 µM was fed into the water reservoir containing either
738 deionized water or 5 mM KNO_3. Transpirational water loss was measured under constant
739 conditions: air humidity of 52.5 %, temperature of 20 °C, and a photon flux density of 500 µmol
740 m^{-2} s^{-1}.

741 **qPCR**

742 Quantitative PCR (qPCR) experiments were performed with samples taken from whole leaves
743 epidermal peels and highly guard cell enriched tissue. Epidermal peels from 12-day-old Barley
744 (cv. Barke) leaves were isolated according to [5]. Thereby only the guard cell subsidiary cell
745 complex survives. For guard cell samples we used the "blender method" on epidermal peels
746 with mature, intact guard cells to mechanically and selectively destroy the subsidiary cells while
747 keeping guard cells alive. Guard cell were enriched within 8 minutes by successive blender
748 cycles (45 seconds each) in ice-cold deionized water with additional crushed ice and filtered
749 through a 210-µm nylon mash. After two rounds of blending, the remaining light green
750 epidermal fraction was further processed. Neutral red staining indicated that at least 90% of the
751 viable cells in the preparations were guard cells. Total RNA from at least three individual
752 biological replicates was prepared using the NucleoSpin® RNA Plant Kit (Macherey Nagel,
753 Drueren, Germany) and stored for subsequent microarray hybridizations or qPCR.

754 For qPCR potential DNA contamination was removed from total RNA by treatment with
755 RNase-Free DNase I (Thermo Scientific, Waltham MA) according to the manufacturer's
756 protocol. First-strand cDNA was prepared using 2.5 µg RNA with the M-MLV-RT kit
757 (Promega, Mannheim, Germany). First-strand cDNA samples were 20-fold diluted in water and
758 subjected to qPCR using a Mastercycler® ep Realplex2S (Eppendorf) with the ABsolute SYBR
759 Capillary Mix (Thermo Scientific, Waltham MA) in 20 µl reaction volumes. Primers used (TIB
760 MOLBIOL, Germany) have been designed according to the sequences from the RNA-seq
761 analyses and validated prior to qPCR. All primers were chosen to amplify fragments not

762 exceeding 500 base pairs. Each transcript was quantified using individual standards. To enable
763 detection of contaminating genomic DNA, PCR was performed with the same RNA as template
764 that was used for cDNA synthesis. Transcripts were each normalized to 10.000 molecules of
765 barley actin4/1. These barley actin fragments, used as house-keeping genes, were homologous
766 to actins 2 and 8 constitutively expressed in most Arabidopsis tissues (for details see [51, 52].
767 All kits were used according to the manufacturer's protocols. The primers are listed in the Key
768 Resources Table.

769 **Energy dispersive X-ray analysis (EDXA)**

770 Leaf samples with open and closed stomata were prepared using the gas exchange setup. Cut
771 leaves were either treated with opening conditions (500 µmol m^{-2} s^{-1} light, 0 ppm CO_2) or
772 closing conditions (darkness, 1000 ppm CO_2) until the transpirational water loss stabilized. The
773 samples were then immediately frozen in liquid nitrogen and lyophilized over a period of 3 days
774 in an ice condenser (Alpha 1-2, Christ GmbH, Germany) under vacuum (R25, Vacuubrand
775 GmbH & CO. KG, Germany) at -55 °C. After the following freeze-drying process leaves were
776 coated with carbon before being examined by a scanning electron microscope (SEM, S-520
777 Hitachi, Tokyo, Japan) equipped with an energy dispersive X-ray device (EDX eumex Si(Li)-
778 detector, EUMEX GV, Mainz, Germany). Single-point measurements on guard cells as well as
779 on subsidiary cells were performed at 10 keV excitation energy, which excites a measurement
780 area of < 2 µm in diameter. Element concentration provided by the analysis data represents the
781 atomic ratio of the analysed ions in percent.

782 **Cloning and cRNA synthesis**

783 The complementary DNAs (cDNAs) of various SLAC1 anion channels, AtSLAH2, AtCPK6,
784 AtABI1 and AtOST1 were cloned into oocyte expression vectors or BiFC expression vectors
785 (both are based on pGEM vectors), by an advanced uracil-excision-based cloning technique as
786 described by [53]. Site-directed mutations were introduced by means of a modified USER
787 fusion method as described by [54, 55]. In brief, the coding sequence of the respective anion
788 channel or kinase within an oocyte expression vector (based on pNBIu vectors, see KEY
789 RESOURCES TABLE) was used as a template for USER mutagenesis. Overlapping primer
790 pairs (overlap covering 8 to 14 bp including the mutagenesis site, see Table S5) were designed
791 [53]. PCR conditions were essentially as described by Nørholm et al (2010, [55]) using PfuX7
792 polymerase. PCR products were treated with the USER enzyme (New England Biolabs,
793 Ipswich, MA, USA) to remove the uracil residues, generating single-stranded overlapping ends.
794 Following uracil excision, recirculation of the plasmid was performed at 37°C for 30 minutes

795 followed by 30 minutes at room temperature, and then constructs were immediately
796 transformed into chemical competent *Escherichia coli* cells (XL1-Blue MRF'). All mutants
797 were verified by sequencing. [54]. The cDNA of Arabidopsis/barley chimeras was also cloned
798 into oocyte expression vectors using a combination of the advanced uracil-excision-based
799 cloning technique and the USER fusion technique [53, 56]. Primers are listed in table S5. For
800 functional analysis, complementary RNA (cRNA) was prepared with the AmpliCap-Max T7
801 High Yield Message Maker Kit (Cellscript, Madison, WI, USA). Oocyte preparation and cRNA
802 injection is described in Experimental Model and Subject Details.

803 **Protein-protein interaction studies (BiFC)**

804 For documentation of the oocyte BiFC results, pictures were taken with a Leica SP5 confocal
805 laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped
806 with multiphoton laser of the Mai Tai-Series (Spectra Physics, Santa Clara, USA) and a Leica
807 HCX IRAPO L25×/0.95W objective.

808 **Oocyte recordings**

809 In double-electrode voltage-clamp studies, oocytes were perfused with Tris/Mes-based buffers.
810 The standard solution contained 10 mM Tris/Mes (pH 5.6), 1 mM $Ca(gluconate)_2$, 1 mM
811 $Mg(gluconate)_2$, 1 mM $LaCl_3$ and 100 mM NaCl, $NaNO_3$ or Na(gluconate). To balance the ionic
812 strength, we compensated for changes in the nitrate or chloride concentration with
813 Na(gluconate). Solutions for anion selectivity measurements were composed of 50 mM malate$^-$
814 , sulphate^{2-}, Cl^-, NO_3^- or gluconate$^-$, 1 mM $Ca(gluconate)_2$; 1 mM $Mg(gluconate)_2$; and 10 mM
815 Tris/Mes (pH 7.5). Osmolality was adjusted to 220 mosmol/kg with D-sorbitol. For recording
816 representative current traces, steady-state currents (I_{SS}) and for calculating the voltage
817 dependent relative open probability (rel. P_O) standard voltage protocol was as follows: Starting
818 from a holding potential (V_H) of 0 mV, single-voltage pulses were applied in 20 mV decrements
819 from +40 to −200 mV. Rel. P_O was calculated from a −120 mV voltage pulse following the test
820 pulses of the standard voltage protocol by fitting the experimental data points with a Boltzmann
821 equation [31] of the form: rel. $P_O = offset + 1 / (1 + \exp (V_{1/2} - V_m) / z)$, where $V_{1/2}$ is the half
822 maximal activation voltage, V_m is the membrane potential and z is the slope of the Boltzmann
823 function. The currents were normalized to the saturation value of the calculated Boltzmann
824 distribution. Instantaneous currents (I_{inst}) were extracted immediately after the voltage jump
825 from the holding potential of 0 mV to 50 ms test pulses ranging from +70 to −150 mV. The
826 reversal potentials (V_{rev}) used for the calculation of the rel. permeability were recorded in the
827 current-clamp mode [18]. For determination of V_{rev} for the respective anion, oocytes were

828 preincubated in 50 mM NO_3^- to gain full activity of the channel. The relative permeability was
829 calculated as described in [36] using the following equation:

830 $\frac{P_X}{P_{NO3}} = \frac{[NO_3^-]_o}{[X^-]_o} e^{\frac{(E_X - E_{NO3})F}{RT}}$ for monovalent anions and $\frac{P_X}{P_{NO3}} = \frac{[NO_3^-]_o}{4[X^{2-}]_o} e^{\frac{(E_X - E_{NO3})F}{RT}} (e^{\frac{-E_X F}{RT}} + 1)$

831 for anions differing in valence (divalent and monovalent). $[NO_3^-]_o$ is the external concentration
832 of the control (nitrate-based) solution and $[X^-]_o$ is the external concentration of the test anion.
833 E_{NO3} is the reversal potential with nitrate and E_X is the reversal potential for the external test
834 anion. F and R are the Faraday and gas constants, respectively, and T is the absolute
835 temperature.

836 To calculate the chord conductance, the reversal potential (V_{rev}) was determined by fitting the
837 instantaneous currents in chloride- and nitrate-containing standard buffers with a linear
838 function. Using the instantaneous currents at –120 mV, the chord conductance could be
839 calculated with the equation $g_{anion} = I_{anion} / (V - V_{rev})$ [31].

840 **Evolutionary coupling analysis**

841 EVfold/EVcouplings (www.evfold.org) [57, 58], a publicly available bioinformatics server,
842 was used to predict evolutionary couplings of the amino acid residues V272 an V273 in
843 AtSLAC1. We used the default transmembrane protein settings and the DI setting as the
844 coupling scoring function. Multiple sequence alignment was done with default settings and
845 resulted in 468 sequences with a e-value cut off of -3. The top 50 amino acid residues (see table
846 S3) that showed evolutionary coupling to AtSLAC1-V272 and V273 were highlighted on
847 previously generated homology models [36] using VMD [43].

Field Code Changed

848 **Frequency logos of TMD1 to 3**

849 Selected SLAC1 homologs were identified by BLASTP (Sequences can be found in table S4).
850 Sequences were aligned using MUSCLE [59] with a gap open penalty of -2.9, gap extend of 0
851 and hydrophobicity multiplier of 5. Transmembrane helix 1, 2 and 3 were identified in the
852 homology model of AtSLAC1 [18] using chimera [60]. Frequency logos were created based on
853 alignments of transmembrane helix 1, 2 and 3 using the weblogo program [61]. Jalview [62]
854 was used to visualize transmembrane helix 1, 2 and 3 alignments.

855 **Alignment, phylogenetic analysis and inference of ancestral SLAC1**

856 *Alignment*

857 Selected SLAC1 homologs were identified by BLASTP. Sequences were aligned using
858 MUSCLE [59] with a gap open penalty of -2.9, gap extend of 0 and hydrophobicity multiplier
859 of 5. Gaps introduced by parts of the sequence supported by 4 or less genes were trimmed.

860 *Phylogenetic analysis*

861 MrBayes 3.2.6 [63] was used to infer the Bayesian phylogenetic tree as previously described
862 [64]. Briefly, Prottest v3.4.2 [65] identified the appropriate LG based phylogenetic model as
863 LG+I+G that use a general amino acid replacement matrix [66] with a proportion of invariable
864 sites (+I) [67] that use a gamma distribution for modelling the rate heterogeneity (+G) [68].
865 Bayesian inference trees were calculated until convergence was reached ("average standard
866 deviation of split frequencies" <0.01). The temperature heating parameter was set to 0.05
867 (temp=0.05) to increase the chain swap acceptance rates, this reduce the chance of Markov
868 chains getting stuck at local high-probability peaks. Burn-in was set to 25% (burninfrac=0.25)
869 and the number of Markov chains was set to 8 (nchains=8).

870 *RAxML*

871 The maximum likelihood phylogenetic tree was inferred using RAxML 8.2.9 as previously
872 described [64]. Briefly, Prottest v3.4.2 [65] identified LG+I+G as the best phylogenetic model.
873 1000 bootstrap replicate searches were performed, and the bootstrap values were portrayed on
874 the MrBayes generated consensus tree when MrBayes values were below 0.95. SLAC1 from
875 *MP* was used as the out-group. All analyses were run in MPI via the CIPRES SCIENCE
876 GATEWAY [69] at the San Diego Supercomputer Center (SDSC). Trees were visualized in
877 figtree (http://tree.bio.ed.ac.uk/software/figtree/) and annotated with Adobe Illustrator.

Field Code Changed

878 *Inference of ancestral sequence*

879 The topology of the phylogenetic tree inferred by MrBayes and RAxML were identical and we
880 used this multiple sequence alignment and the phylogenetic tree as input for inference of the
881 ancestral sequence of the last common ancestor of monocots and dicots. Probabilities for the
882 ancestral sequence at the split between monocots and dicots (figure S4A) was calculated using
883 the LG model of substitution by FastML [42]. The top 100 most likely sequences showed a log
884 likelihood difference of only 0.19 which suggests that sequence #1 and #100 are almost as likely
885 to be true. The N- and C-terminus of SLAC1 has activating/regulatory roles and thus we,
886 respectively, substituted the residues 1-182 and 514-556 from the AncSLAC1 N- and C-
887 terminal residues with the corresponding residues from AtSLAC1 (for sequence information

888 see table S4). We resurrected AncSLAC1 by GeneStrand synthesis (Eurofins Medigenomix
889 GmbH, Campus Ebersberg, Germany).

890

891 **QUANTIFICATION AND STATISTICAL ANALYSES**

892 All experiment was performed at least two times (independent experiments). Sample size, n,
893 and statistical details (mean ± standard error, SE or standard deviation, SD) for each experiment
894 are given in the figure legends. Statistical significances based on one-way ANOVA. For
895 statistical analysis the software Igor Pro7 (waveMetrics, Inc., Lake Oswego, Oregon, USA),
896 Excel (Microsoft Corp. Redmond, Washington, USA) was used.

897

898 **DATA AND SOFTWARE AVAILABILITY**

899 Raw RNA-seq sequence reads are available at the European Nucleotide Archive with accession
900 number ArrayExpress accession E-MTAB-5877.

901 Accession numbers: HvSLAC1 (*Hordeum vulgare* cultivar Barke); AtSLAC1 (*Arabidopsis*
902 *thaliana* Col-0) At1g12480; OsSLAC1 (*Oryza sativa* Japonica Group) XP_015636891;
903 SlSLAC1 (*Solanum lycopersicum*) XP_004245686; NtSLAC1 (*Nicotiana tabacum*)
904 XP_016515379; ZomSLAC1 (*Zostera marina*) KMZ58505; PdSLAC1 (*Phoenix dactylifera*)
905 XP_008780343.1; PpSLAC1 (*Physcomitrella patens*) PNR63146.1; CrSLAC1a (*Ceratopteris*
906 *richardii*) KT238910; SmSLAC1b (*Selaginella moellendorffii*) KU556809; AtOST1
907 (*Arabidopsis thaliana* Col-0) At4g33950; AtCPK6 (*Arabidopsis thaliana* Col-0) At2g17290;
908 AtABI1 (*Arabidopsis thaliana* Col-0) At4g26080.

909 Software and algorithms used in this study are listed in the KEY RESOURCES TABLE. In
910 addition, for graph preparations and statistical analysis the software Igor Pro7 (waveMetrics,
911 Inc., Lake Oswego, Oregon, USA), Excel (Microsoft Corp. Redmond, Washington, USA),
912 Adobe Illustrator (Adobe Systems Incorporated, San Jose, California, USA) and CorelDRAW
913 (Corel Corporation, Ottawa, Ontario, Canada) was used.

914 **Supplemental tables**

915 **Table S1: Differentially expressed genes (DEG) in guard cell complexes (related to figure**
916 **S1).**

917 **Table S2: Selection of transcripts that are involved in stomatal movement (related to**
918 **figure S1).**

919 **Table S3: The top 50 amino acid residues that showed evolutionary coupling (EC) to**
920 **AtSLAC1 V272 and V273 are shown here (related to figure 4).**

921 **Table S4: Gene names, species and SLAC1 amino acid sequences that were used to build**
922 **alignments, frequency logos and phylogenetic trees (related to figure 4, figure S3 and S4).**

923 **Table S5: Oligos used in this study (related to figure S1 and METHODS DETAILS).**