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► To cite this version:

Jing Zhou, Thanh-Hao Nguyen, Dorsaf Hmidi, Doan-Trung Luu, Hervé Sentenac, et al.. The outward shaker channel OsK5.2 improves plant salt tolerance by contributing to control of both leaf transpiration and K^+ secretion into xylem sap. *Plant, Cell and Environment*, 2022, 45 (6), pp.1734-1748. 10.1111/pce.14311 . hal-03683360

HAL Id: hal-03683360

<https://hal.inrae.fr/hal-03683360>

Submitted on 9 Aug 2022

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1 The outward Shaker channel OsK5.2 improves plant salt tolerance by contributing to control
2 of both leaf transpiration and K⁺ secretion into xylem sap

3

4 Running head: OsK5.2 K⁺ channel contribution to salt tolerance

5

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13

14 **Funding**

15 This work was supported in part by a grant from the China Scholarship Council (to J.Z.), by a
16 doctoral fellowship from the French Embassy in Vietnam (to T.H.N.), and by an ANR-DFG
17 grant (ANR-20-CE92-0005 to A.A.V.).

18

19 **Acknowledgments**

20 We are grateful to Emmanuel Guiderdoni, Christian Chaine, Eve Lorenzini, Christophe Périn
21 and Remy Michel (UMR AGAP, Montpellier) for the rice mutant line amplification, and to
22 Aurélie Putois (UMR IATE, Montpellier) for assistance with picture acquisitions at the
23 Scanning Electron Microscope.

24

25 **Abstract**

26 Soil salinity constitutes a major environmental constraint to crop production worldwide. Leaf
27 K^+/Na^+ homeostasis, which involves regulation of transpiration, and thus of the xylem sap
28 flow, and control of the ionic composition of the ascending sap, is a key determinant of plant
29 salt tolerance. Here, we show, using a reverse genetics approach, that the
30 outwardly-rectifying K^+ -selective channel *OsK5.2*, which is involved in both K^+ release from
31 guard cells for stomatal closure in leaves and K^+ secretion into the xylem sap in roots, is a
32 strong determinant of rice salt tolerance (plant biomass production and shoot phenotype
33 under saline constraint). *OsK5.2* expression was up-regulated in shoots from the onset of the
34 saline treatment, and *OsK5.2* activity in guard cells led to fast decrease in transpirational
35 water flow and therefore reduced Na^+ translocation to shoots. In roots, upon saline treatment,
36 *OsK5.2* activity in xylem sap K^+ loading was maintained, and even transiently increased,
37 outperforming the negative effect on K^+ translocation to shoots resulting from the reduction
38 in xylem sap flow. Thus, the overall activity of *OsK5.2* in shoots and roots, which both
39 reduces Na^+ translocation to shoots and benefits shoot K^+ nutrition, strongly contributes to
40 leaf K^+/Na^+ homeostasis.

41

42 **Keywords (5-10)**

43 Outward K^+ channel, Shaker channel, salt tolerance, rice, K^+/Na^+ homeostasis, transpirational
44 flux, xylem sap, root-to-shoot translocation, *Tos17* insertion mutants

45

46

47 **1 | INTRODUCTION**

48 High soil salinity is a widespread environmental constraint over the world that causes
49 substantial restrictions in production and quality of a majority of crops, including cereals.
50 Understanding how plants cope with high salinity in the environment is thus an issue of great
51 agricultural importance. Rice is rated as a salt-sensitive cereal (Munns & Tester, 2008; Zeng
52 & Shannon, 2000), and salinity levels have increased in rice fields, particularly due to the
53 climate change and sea level rise, which strongly challenge rice culture in coastal regions.

54 The adverse effects of high soil salinity on plant growth are mainly related to the
55 decrease in osmotic potential of the soil and to ionic toxicity of Na^+ in leaves (Munns &
56 Tester, 2008). Studies on the latter phenomenon have revealed that genes encoding Na^+
57 transport systems correspond to major quantitative trait loci (QTLs) of salt tolerance (Hauser
58 & Horie, 2010). In return, such findings have spurred research efforts in this domain of
59 membrane transport biology, highlighting also that Na^+ detrimental effects are counteracted
60 by the plant's ability to take up the essential macronutrient K^+ and control its K^+ nutritional
61 status in presence of high external Na^+ concentrations (Maathuis & Amtmann, 1999). K^+ is
62 involved in vital functions such as enzyme activation, the cytoplasmic pH homeostasis,
63 control of cell membrane potential and cell turgor-driven movements (Marschner, 2011;
64 Nieves-Cordones, Al Shiblawi & Sentenac, 2016). Upon salt stress, the massive influx of
65 positively charged Na^+ ions causes cell membrane depolarization, which reduces the driving
66 force for K^+ uptake and even in some cases leads to channel-mediated root K^+ losses
67 (Jayakannan, Bose, Babourina, Rengel, & Shabala, 2013; Rubio, Nieves-Cordones, Horie, &

68 Shabala, 2020). Thus, plant exposure to high salinity is inevitably accompanied by chronic
69 K^+ deficiency, which affects the leaf K^+ to Na^+ content ratio, whose maintenance to a high
70 value is a key determinant of salt tolerance (Hauser & Horie, 2010; Maathuis & Amtmann,
71 1999).

72 K^+ and Na^+ ions taken up by root cells can migrate to stelar tissues and be translocated to
73 leaves by the upward flow of sap in the xylem vessels. Control of the ionic composition of
74 xylem sap, involving membrane ion transport processes in parenchyma cells along the sap
75 ascent pathway, is thus a major determinant of salt tolerance, together with control of the flux
76 of xylem sap, which is driven by leaf transpiration and hence dependent on the level of
77 stomatal aperture, or driven by the so-called root pressure, resulting from increased osmotic
78 pressure in the xylem vessels due to increased solute concentration in the sap in absence of
79 significant plant transpiration (Jeschke, 1984; Marschner, 2011). Therefore, transport systems
80 contributing to Na^+ or K^+ secretion/retrieval into/from the xylem sap or to regulation of
81 stomatal aperture can contribute to processes that play crucial roles in salt tolerance.

82 In various plant species, Na^+ transporters from the HKT family have been shown to
83 contribute to Na^+ retrieval from the xylem sap and loading into the xylem parenchyma cells
84 bordering the vessels, *i.e.* to the so-called "sap desalinization" process (Hauser & Horie,
85 2010). In rice, the *HKT* transporter genes identified as involved in this process are *OsHKT1;5*,
86 which is mainly expressed in root xylem parenchyma cells and corresponds to the major
87 salt-tolerance QTL *SKCI* (Ren et al., 2005), *OsHKT1;4*, expressed in both root and basal leaf
88 xylem tissues (Suzuki et al., 2016; Khan et al., 2020), and *OsHKT1;1* expressed in both
89 xylem and phloem and thereby also involved in Na^+ recirculation from leaves to roots within

90 the phloem sap, thereby making an additional contribution to promoting Na⁺ accumulation in
91 roots compared to leaves (Campbell et al., 2017; Wang et al., 2015).

92 Compared with the large number of studies focused on the Na⁺ transporters controlling
93 Na⁺ translocation to leaves and thereby contributing to maintain the ratio of leaf K⁺ to Na⁺
94 contents to a high value, less attention has been paid to the K⁺ transport mechanisms that
95 operate under saline conditions and ensure efficient K⁺ supply to leaves. Current knowledge
96 in this domain essentially concerns K⁺ transport systems involved in root K⁺ uptake, and
97 mainly high-affinity K⁺ transporters from the HAK/KUP/KT family, AtHAK5 in Arabidopsis
98 and OsHAK1, OsHAK5, OsHAK16 and OsHAK21 in rice (Chen et al., 2015; Feng et al.,
99 2019; Nieves-Cordones, Alemán, Martínez & Rubio, 2010; Shen et al., 2015; Yang et al.,
100 2014).

101 In rice, the outwardly rectifying Shaker K⁺ channel OsK5.2 is involved both in K⁺
102 secretion into the xylem sap for translocation toward the shoots and in control of stomatal
103 aperture and leaf transpiration by driving K⁺ efflux from guard cells for stomatal closure
104 (Nguyen et al., 2017). OsK5.2 is homologous to two outwardly rectifying K⁺ channels from
105 Arabidopsis, SKOR and GORK, respectively involved in K⁺ secretion into the xylem sap
106 (Gaymard et al., 1998) and K⁺ efflux from guard cells for stomatal closure (Hosy et al., 2003).
107 Gathering the two functions of its Arabidopsis counterparts, OsK5.2 emerged as a good
108 model to assess the level of contribution of these functions to the control of Na⁺ and K⁺
109 delivery to shoots upon saline conditions and salt tolerance. This has been achieved in the
110 present study by phenotyping *osk5.2* knock-out (KO) mutant plants subjected to saline
111 conditions. We found that the lack of functional *OsK5.2* expression does result in increased

112 plant sensitivity to salt stress and analyzed the bases of the salt sensitive phenotype.

113

114 **2 | MATERIALS AND METHODS**

115 **2.1 | Plant growth and salt treatment**

116 The selection from *Tos17*-insertion lines of *osk5.2* mutant and corresponding wild-type (WT)
117 plants in the background of rice Nipponbare cultivar (*Oryza sativa* L. ssp. japonica cv.
118 Nipponbare) has been previously described (Nguyen et al., 2017). Rice seeds were
119 germinated on a raft floating on deionized water for one week. The seedlings were then
120 hydroponically grown on half-strength Yoshida medium for one week, and thereafter on
121 Yoshida medium (0.5 mM (NH₄)₂SO₄, 1.6 mM MgSO₄, 1.2 mM Ca(NO₃)₂, 0.7 mM KNO₃,
122 0.8 mM KH₂PO₄, 60 μM Na₂FeEDTA, 20 μM MnSO₄, 0.32 μM (NH₄)₆Mo₇O₂₄, 1.4 μM
123 ZnSO₄, 1.6 μM CuSO₄, 45.2 μM H₃BO₃, and pH adjusted to 5.5 with H₂SO₄). Five-week-old
124 rice plants were subjected to salt treatment by supplementing the hydroponic Yoshida
125 medium with 50 mM NaCl for up to 14 days (and also 100 mM for 7 days in growth assays).
126 The rice plants were grown in a growth chamber (70% relative humidity, light intensity 130
127 photon μmol.m⁻².s⁻¹, 29°C/25°C 12 h/12 h day/night).

128

129 **2.2 | RNA extraction and quantitative real time PCR experiments**

130 Five-week-old Nipponbare plants grown on Yoshida medium were either supplemented with
131 50 mM NaCl for 14 days and then transferred back to standard Yoshida medium for three
132 days, or further grown during this period on Yoshida medium (control batch). Total RNAs
133 were extracted from samples collected at same times from salt-treated or control plant

134 batches using the RNeasy plus mini kit with gDNA eliminator (Qiagen, Germany).
135 First-strand cDNAs were synthesized from 3 μg of RNAs using SuperScript III reverse
136 transcriptase (Invitrogen) and used as template for qRT-PCR experiments. qRT-PCR
137 analyses were performed using the Lightcycler480 system (Roche diagnostics) and SYBR
138 *Premix Ex Taq* (Takara) in a total volume of 10 μl , which contained 2 μl of cDNA, 3 μl of
139 forward and reverse primer mixture (1 μM), and 5 μl of SYBR *Premix Ex Taq*. Reactions
140 were performed with three independent biological replicates, each one with three technical
141 replicates (PCR program: 95°C for 30 sec; 45 cycles of 95°C for 10 sec, 60°C for 10 sec, and
142 72°C for 15 sec; followed by a melt cycle from 60°C to 95°C). C_T (cycle threshold) values
143 were obtained from amplification data using a threshold of 0.37. The *OsK5.2* absolute
144 number of copies was calculated according to standard curves obtained by successive
145 dilutions with known quantities of *OsK5.2*, and then normalization using the C_T values of
146 three housekeeping genes (ubiquitin-like protein gene *SMT3*, PP2A-interactor gene *Tip41* and
147 elongation factor gene *EF1beta*) as described in Khan et al. (2020). The sequences of the
148 primers used for qRT-PCR experiments are provided in Table S1.

149

150 **2.3 | Na⁺ and K⁺ assays in tissues and xylem sap**

151 Five-week-old plants hydroponically grown as described above were supplemented or not
152 with 50 mM NaCl for 14 days. Excised root systems and shoots were periodically collected
153 during the salt treatment, both from salt treated and control plants. The roots (rinsed in
154 deionized water) and shoots were dried (60°C for 3 days) and weighed. Ions were extracted
155 from the tissues in 0.1 N HCl for 3 days and assayed by flame spectrophotometry (SpectrAA

156 220FS, Varian).

157 Xylem sap was collected through natural exudation under control condition from
158 de-topped plants (3 cm above the root system). Upon salt treatment, xylem sap was obtained
159 through pressurization. The root system of the de-topped plants was placed into a pressure
160 chamber (Boursiac *et al.*, 2005) filled with hydroponic medium containing 50 mM NaCl, and
161 sealed with a silicon dental paste (PRESIDENT Light Body, Coltene, Switzerland). The first
162 few drops (2 μ l) were discarded to avoid the contamination that results from injured cells.
163 Twenty μ l of sap samples were then collected using a micro-pipette, transferred into 0.2 ml
164 Eppendorf tubes kept on ice, and diluted in 0.1 N HCl for Na⁺ and K⁺ assay (flame
165 spectrophotometry).

166

167 **2.4 | Leaf transpiration**

168 Intact plants were transferred into a multipotometer device (Nguyen *et al.*, 2017) one day
169 before transpiration rate measurement. The root system of each intact plant was inserted into
170 a 50 ml syringe filled with hydroponic medium through the rubber plunger, and sealed with a
171 silicon dental paste. Each syringe was connected to a graduated 1-ml serological plastic
172 pipette via a thin silicone tube. The pipettes were refilled with the same medium with proper
173 time intervals, making sure that no air bubble was present in the system. A camera took
174 photographs of the set of pipettes every two minutes in order to record the changes in water
175 level in the pipettes. Image capture was started after 3 h under light condition and maintained
176 for 30 minutes. Plants were then exposed to darkness for 5 h under continuous recording. The
177 rate of decrease in the water volume in the pipettes was used to calculate the mean

178 transpiration rate of plants. After transpiration rate measurements, each plant was placed on a
179 paper sheet of known dimension, the blades being thoroughly positioned with small pieces of
180 double-sided tape in order to fix the abaxial face on the paper sheet and to avoid blade
181 superposition. A picture of the plant was then taken and the whole area of leaf tissues was
182 determined using imageJ software (imagej.nih.gov/ij) for transpiration rate value
183 normalization.

184

185 **3 | RESULTS**

186 **3.1 | *osk5.2* mutant plants exhibit increased sensitivity to salinity**

187 The two *osk5.2* KO mutant lines ASJA08 and ASHF06 (Nguyen et al., 2017) and the
188 corresponding wild type (WT) plants displayed a very similar development and phenotype
189 when growth occurred in absence of salt stress (Figure 1). This was no longer the case when
190 the plants were subjected to saline conditions (6-week-old plants subjected to 50 or 100 mM
191 NaCl for 7 days). The mutant plants then displayed a severe reduction in biomass production
192 when compared with that of plants from the same mutant lines but grown in control
193 conditions, by about 25-35% with 50 mM NaCl and 45-50% with 100 mM NaCl (Figure 1a,
194 b). Conversely, the corresponding reduction observed for the WT plants appeared weak (~2%
195 with 50 mM NaCl and less than 10-15% with 100 mM NaCl) and not statistically significant
196 (Figure 1b). While the biomass of mutant plants in control conditions was similar to that of
197 corresponding wild type plants, the length of leaves and roots were significantly reduced in
198 mutant plants, only slightly in leaves (by ~10%) but more in roots (by 15-20%)(Figure S1).
199 The saline treatments however, especially stunted leaf growth in the mutant plants, increasing

200 the leaf length difference between mutant and wild type plants to 20% with 100 mM NaCl
201 (Figure S1). The saline treatments had no differential effect on root length between mutant
202 and wild type plants, on the other hand (Figure S1). Shoot water content decreased slightly
203 with the most severe salt treatment but, whatever the treatment, no significant difference in
204 shoot or root water content was found between mutant and wild-type plants (Figure S2).
205 More “dried” leaf tips in the mutant than the WT plants were however observed under the
206 most severe salt treatment (Figure 1a). Altogether, these analyses indicated that the lack of
207 *OsK5.2* functional expression resulted in reduced tolerance to saline conditions.

208

209 **3.2 | *OsK5.2* transcript accumulation under salt stress**

210 Real-time qRT-PCR analyses revealed that *OsK5.2* was expressed in both roots and leaves
211 (Figure 2). In plants grown in control condition, *OsK5.2* transcripts were 4 folds more
212 abundant in roots than in leaves. When the plants were subjected to saline conditions (50 mM
213 NaCl), a change in the relative expression of *OsK5.2* between roots and shoots was observed
214 leading to balanced expression in the plant. In leaves, the accumulation of *OsK5.2* transcripts
215 was rapidly (from one day after salt treatment) up-regulated by about 3 folds, and remained
216 high during 7 days (Figure 2b). The leaf level of *OsK5.2* transcripts appeared to decrease
217 with longer exposure to the saline conditions but, after 14 days of salt treatment, it was still
218 about 1.5-fold that observed in control conditions (Figure 2b). Recovery from salt stress for 1
219 to 3 days further decreased the leaf level of *OsK5.2* transcripts, down to that observed in
220 leaves from control plants. In roots, as compared with leaves, the accumulation of *OsK5.2*
221 transcripts showed less variation in response to the saline treatment and tended to slightly

222 decrease throughout the saline treatment period, a down-regulation by about 25% being
223 statistically significant at days 3 and 14 of the treatment (Figure 2a). After 1 day of recovery
224 from salt stress, the root level of *OsK5.2* transcripts recovered to same expression level of
225 control plants and then remained stable for at least 2 days.

226

227 **3.3 | *osk5.2* mutant plants display larger transpirational water loss than WT plants** 228 **under salt stress**

229 Stomatal aperture measurement is hardly feasible in rice due to the small size of rice stomata
230 and the dumbbell-shaped guard cells, which lead to extremely thin aperture of the stomatal
231 pore (Figure S3; Huang et al., 2009; Matsuda et al., 2016). Absence of noticeable effect of
232 *OsK5.2* loss-of-function on stomatal shape was found on the abaxial side of the flag leaf
233 using scanning electron microscopy (Figure S3). The role of *OsK5.2* in control of leaf
234 transpiration under salt stress was then investigated by adding 50 mM NaCl into the
235 hydroponics solution of 5-week-old *osk5.2* mutant and WT plants, and performing
236 measurements of the steady-state rates of plant water loss periodically for 14 days (on days 1,
237 3, 7 and 14 of the salt treatment) both under light and in dark conditions using a
238 multipotometer (Figure 3). The data obtained with the 2 *osk5.2* mutant lines ASJA08 and
239 ASHF06 led to the same conclusions. The salt treatment strongly decreased the rate of
240 transpirational water loss in the *osk5.2* mutant and the corresponding WT plants, by up to 50
241 to 55 % in the light and 30 to 40% in the dark conditions (Figure 3). The kinetics of reduction
242 of transpiration rate upon NaCl exposure was clearly slower in *osk5.2* mutant compared with
243 WT plants both in light and dark conditions. During the two weeks of salt treatment, a higher

244 rate of transpirational water loss was consistently observed in *osk5.2* mutant as compared
245 with WT plants in light and dark conditions, and the difference was highly significant in most
246 of the analyzed time points (Figure 3). The greatest difference in transpiration rate between
247 WT and *osk5.2* mutant plants occurred after one day of treatment owing to the slower
248 response to NaCl exposure in *osk5.2* mutant plants.

249

250 **3.4 | Na⁺ and K⁺ concentrations in xylem sap under salt stress and translocation fluxes** 251 **towards the shoots**

252 Xylem sap samples were collected from de-topped plants subjected to the same protocol of
253 salt treatment as that used in the experiment described by Figure 3: 2 weeks in 50 mM NaCl
254 hydroponics solution applied to 5-week-old plants previously grown under control conditions.
255 The concentrations of K⁺ and Na⁺ determined in sap samples (Figure 4) and the transpiration
256 rates recorded under the same experimental conditions (Figure 3) were used to estimate the
257 transpiration-driven fluxes of K⁺ and Na⁺ (Figure 5) arriving in shoots under such conditions.

258 Under control condition, K⁺ concentrations measured in xylem sap were close to 11 mM
259 (7-folds the K⁺ concentration in the hydroponic medium) in WT plants, and were 30 to 40%
260 lower in the two *osk5.2* mutant lines (as previously reported; Nguyen et al., 2017). The xylem
261 sap K⁺ concentration in both WT and *osk5.2* mutant plants displayed transient variations
262 upon exposure to the saline conditions (Figure 4a). The concentrations observed in the *osk5.2*
263 mutant plants remained lower than those displayed by the corresponding WT plants by about
264 40% to 50% over the entire duration of the salt treatment (Figure 4a). With respect to Na⁺,
265 the concentration of this cation in the xylem sap was extremely low, in the submillimolar

266 range, in the absence of salt treatment (Figure 4b). Exposure to 50 mM NaCl led to the
267 loading of a large amount of Na⁺ into the xylem sap with no significant difference between
268 WT and *osk5.2* mutant plants (Figure 4b). The Na⁺ concentrations measured in the xylem sap
269 of the two types of plants were close to that in the hydroponic medium (50 mM) after one day
270 of NaCl supplementation, and remained fairly stable during the two weeks of salt treatment.
271 Altogether, these results indicated that the lack of *OsK5.2* functional expression
272 constitutively resulted in a large reduction in xylem sap K⁺ concentration, by *ca.* 40-50%, but
273 did not affect xylem sap Na⁺ concentration. As a result, the K⁺/Na⁺ xylem sap concentration
274 ratios, computed from the data provided by Figure 4a and 4b, appear consistently higher in
275 WT than in *osk5.2* mutant plants (Figure 4c).

276 The estimated K⁺ flux arriving in shoots during the light period in these experimental
277 conditions, obtained by integrating the data from Figure 3a and 4a (Figure 5a), is decreased
278 by the salt treatment in both the *osk5.2* mutant and WT plants. It is lower in both *osk5.2*
279 mutant lines than in the corresponding WT plants, except at day 1 of the salt treatment due to
280 the sharp decrease in transpiration rate displayed by WT plants at this time point. The
281 differences between the WT and mutant plants under salt treatment are in the 30 to 50%
282 range from day 3 of the salt treatment, *i.e.*, similar to those observed between the two types of
283 plants in control conditions (Figure 5a).

284 The estimated flux of Na⁺ arriving in the shoots of WT and *osk5.2* mutant plants is very
285 low under control conditions (this cation being then present as trace contaminant in the
286 hydroponics solution). A marked increase in Na⁺ flux is observed in all genotypes from the
287 first day of exposure to NaCl (Figure 5b). The flux is larger in *osk5.2* mutant than in WT

288 plants, by ca. 20 to 40% during the first week of the salt treatment, which essentially reflects
289 the difference in transpiration rate between the two types of plants during this period (see
290 Figure 3).

291

292 **3.5 | *osk5.2* mutant plants accumulate less K⁺ and more Na⁺ under salt stress**

293 K⁺ and Na⁺ contents were determined in roots and shoots of *osk5.2* and WT plants subjected
294 to the same salt treatment protocol as that previously used.

295 In all genotypes, root and shoot K⁺ contents, and thus whole plant K⁺ contents, decreased
296 with the duration of the salt treatment (Figure 6a, b, respectively). In shoots, the decrease was
297 clearly more pronounced in the *osk5.2* mutant lines, when compared with the corresponding
298 WT plants, and the relative difference in shoot K⁺ contents between the mutant and WT
299 plants increased with the duration of the salt treatment (Figure 6b): the difference was in the
300 range of 10-20% at the beginning of the treatment (in the absence of NaCl addition and at day
301 1 of the salt treatment), and reached 40-45% after two weeks of treatment (Figure 6b). In
302 roots, the impact of lacking *OsK5.2* functional expression on K⁺ contents appeared much
303 weaker than that observed in shoots, mostly not statistically significant (Figure 6a).

304 Regarding Na⁺, the contents of this cation were very low in all plants, whatever their
305 genotype, in the absence of salt treatment (Figure 7). The salt treatment increased both the
306 root and shoot (and thus the whole plant) contents of this cation, from the first day of
307 treatment and over the two weeks of treatment, in all plant genotypes (Figure 7). Under all
308 conditions except the longest duration of the salt treatment (in other words, under control
309 conditions and during the first week of salt exposure), significantly higher Na⁺ contents were

310 found in the *osk5.2* mutants than in the corresponding WT plants, for both mutant lines, by
311 more than 20% in roots (Figure 7a) and 35% in shoots (Figure 7b). The relative differences
312 between the mutant and WT plants were weaker at the last time point of salt treatment (after
313 14 days), and the differences were no longer statistically significant (except for the roots of
314 one mutant line). At this time, Na⁺ levels in shoots exceeded those in roots in all genotypes
315 (Figure 7a, b). Thus, Na⁺ accumulation was higher in *osk5.2* mutant plants than in the
316 corresponding WT plants until the late stage of Na⁺ plant invasion, when the level of Na⁺ in
317 shoots had become higher than that in roots.

318 K⁺/Na⁺ content ratios were calculated from the data displayed by Figures 6 and 7. The
319 ratios were significantly lower in *osk5.2* mutant plants than in WT plants both in shoots and
320 roots under all conditions except in roots of one mutant line at the last time point of salt
321 treatment (Figure 8). In salt-stressed leaves, the relative reduction in K⁺/Na⁺ content ratios
322 observed in the *osk5.2* mutant plants as compared with the corresponding WT plants, was in
323 the range of 35-55% (Figure 8). Thus, *OsK5.2* lack of functional expression strongly
324 impaired K⁺/Na⁺ homeostasis in leaves under salt stress.

325

326 **4 | DISCUSSION**

327 **4.1 | OsK5.2, a model for assessing the involvement of K⁺ channels in the control of K⁺ 328 and Na⁺ translocation to the shoots under salt stress**

329 Mechanisms that control long distance transport of Na⁺ and K⁺ in the plant vasculature
330 contribute to maintaining the shoot K⁺/Na⁺ content ratio at a high value, which is a key
331 determinant of salt tolerance (Munns and Tester, 2008; Maathuis, Ahmad and Patishtan, 2014;

332 Ismail and Horie, 2017; Wu, Zhang, Giraldo and Shabala, 2018).

333 Regarding Na⁺, clear evidence has been obtained, in rice, as well as in Arabidopsis and
334 various other species, that Na⁺ transporters belonging to the HKT family are involved in
335 desalinization of the ascending xylem sap (Hauser & Horie, 2010). The H⁺/Na⁺ antiport
336 system SOS1 has been suggested to also contribute to this function when the concentration of
337 Na⁺ in the xylem sap reaches very high values (Maathuis et al., 2014).

338 Regarding the mechanisms controlling K⁺ translocation to shoots, outwardly rectifying
339 channels belonging to the Shaker family (SKOR in Arabidopsis and OsK5.2 in rice; see
340 below), have been shown to mediate K⁺ secretion into the xylem sap under normal conditions
341 (Gaymard et al., 1998; Nguyen et al., 2017) but their contribution to this function under salt
342 stress remains poorly documented. Furthermore, K⁺ secretion into the xylem sap may have to
343 be active, under some environmental conditions (Wu, Zhang, Giraldo and Shabala, 2018),
344 which would exclude channel-mediated (passive) contribution to this function in such
345 conditions. Also, active H⁺-coupled K⁺ transports mediated by HAK/KUP/KT transporters
346 (see below) or involving a NRT1/PTR family member, NRT1;5 (Li et al., 2017), have been
347 shown to contribute to K⁺ translocation towards the shoots. In rice, OsHAK1 and OsHAK5,
348 which are thought to be endowed with H⁺-K⁺ symport activity (Véry et al., 2014), have been
349 shown to play a role in K⁺ translocation towards the shoots under saline conditions (Chen et
350 al., 2015; Yang et al., 2014). The mechanisms that underlie these contributions remain
351 however to be specified. H⁺-K⁺ symport activity in parenchyma cells bordering the xylem
352 vessels would result in K⁺ retrieval from the xylem sap since the pH gradient between the sap
353 and the cytoplasm is inwardly directed and thus favors K⁺ influx into the cells. It has thus

354 been hypothesized that such H⁺-K⁺ symporters may allow K⁺ acquisition within the stele by
355 parenchyma cells, and that this would result in a higher concentration of K⁺ in
356 xylem-adjacent cells, and thus in an outwardly-directed K⁺ electrochemical gradient that
357 would allow (passive) channel-mediated K⁺ release into the sap (Yang et al., 2014).

358 At the leaf surface, control of stomatal aperture (and hence of transpiration rate) provides
359 another type of contribution to salt tolerance. Exposure to saline conditions has been shown
360 to rapidly result in stomatal closure, which limits the flux of xylem sap, and thus the rate of
361 Na⁺ translocation to shoots (Fricke et al., 2006; Huang et al., 2009; Hedrich and Shabala,
362 2018). Such a control is however likely to also affect the rate of K⁺ translocation to shoots,
363 and thus its contribution to shoot K⁺/Na⁺ homeostasis should benefit from mechanisms
364 allowing to counteract the depressive effect of the reduction in volumetric flow of xylem sap
365 on K⁺ translocation (see § 4.4).

366 The two outwardly rectifying Shaker K⁺ channels in Arabidopsis, SKOR and GORK,
367 are involved in xylem sap K⁺ loading (Gaymard et al., 1998), and in guard cell K⁺
368 release-mediated stomatal closure (Hosy et al., 2003), respectively. The roles of SKOR and
369 GORK in Arabidopsis salt tolerance remain, however, poorly documented. OsK5.2, which is,
370 like SKOR and GORK, an outwardly rectifying Shaker channel, had been previously shown
371 to be involved both in xylem sap K⁺ loading and in guard cell K⁺ release-mediated stomatal
372 closure (Nguyen et al., 2017). The expression level of *OsK5.2* is fairly maintained in roots
373 under saline conditions, and even increased in shoots in these conditions (Figure 2). This
374 channel has thus been used as a model in the present report to investigate xylem sap K⁺
375 loading under salt stress, i.e., whether it can be channel mediated or requires active transport

376 systems (e.g., H⁺/K⁺ antiporters?), and the involvement of transpiration rate control in salt
377 tolerance.

378

379 **4.2 | K⁺ Secretion into the xylem sap under salt stress**

380 Saline conditions weakly affected the expression level of *OsK5.2* in roots (Figure 2a), as
381 shown for its counterpart *SKOR* in Arabidopsis roots (Pilot et al., 2003). In line with this
382 rather stable expression, the contribution of *OsK5.2* to K⁺ secretion into the xylem sap
383 (estimated from the difference in sap concentration between the WT and *osk5.2* mutant plants;
384 Figure 4a) did not appear to be much modified by the salt treatment. This contribution even
385 tended to slightly increase during the first week of the treatment, which may be due to
386 increased driving force for K⁺ secretion under conditions of salt-induced membrane
387 depolarization (Jayakannan et al., 2013; Mian et al., 2011).

388 Reliable measurements of both the membrane potential and the apoplastic K⁺
389 concentration of stelar cells are difficult to obtain. However, the fact that *OsK5.2* can
390 contribute to K⁺ secretion under salt stress provides definitive evidence that passive (since
391 channel-mediated) secretion of K⁺ can occur in stelar cells of rice plants facing saline
392 conditions. This conclusion, which does not exclude a contribution of active K⁺ transport
393 mechanisms to K⁺ secretion under saline conditions, also means that other channels besides
394 *OsK5.2*, either K⁺-selective and belonging to the Shaker family (Véry et al., 2014) or poorly
395 K⁺-selective like NSCC channels identified in stelar cells by patch clamp experiments
396 (Wegner & de Boer, 1997), could also contribute to K⁺ secretion into xylem sap under such
397 conditions.

398

399 **4.3 | Reduction of the volumetric flux of xylem sap under salt stress**

400 Exposure to saline conditions is known to rapidly result in reduced stomatal aperture and
401 plant transpiration (Fricke et al., 2006; Hedrich & Shabala, 2018; Robinson, Véry, Sanders,
402 & Mansfield, 1997). In the present study, the transpiration rate was similarly reduced in WT
403 and *osk5.2* mutant plants at the end of the salt treatment, by about 50% under light conditions
404 and 30% under dark conditions (Figure 3). The kinetics of the reduction in transpiration rate
405 was however more rapid in WT than in *osk5.2* mutant plants.

406 Upon an increase in external medium salinity, abscisic acid (ABA) produced in response
407 to the resulting osmotic stress is rapidly directed to guard cells, where it is expected to
408 activate the PYR/PYL/RCAR-ABI1 PP2C phosphatase-OST1 SnRK kinase signaling
409 pathway, leading to guard cell anion channel activation and stomatal closure (Hedrich &
410 Shabala, 2018). The actual contribution of guard cell anion channels to the triggering of
411 stomatal closure upon salt stress has however been little investigated so far. Likewise, the
412 role in stomatal closure upon salt stress of the K⁺ outward channels acting as downstream
413 effectors (Pandey et al., 2007; Schroeder et al., 2001) was still poorly documented. Indeed,
414 although extensive analyses have concerned the integrated involvement of transport systems
415 in regulation of guard cell turgor (Jezek & Blatt, 2017), little information is related to high
416 salinity conditions (Lebaudy et al., 2008; Thiel & Blatt, 1991; Véry, Robinson, Mansfield, &
417 Sanders, 1998). Here, our data reveal the important role of channel-mediated outward K⁺
418 transport activity in guard cells in control of transpiration rate upon salt stress. The whole
419 plant transpiration data (Figure 3) suggest that OsK5.2 activity in stomata contributed to the

420 reduction in stomatal aperture over the entire duration of the salt treatment. Furthermore, our
421 data indicate that this activity is of particular importance at the onset of salt stress by allowing
422 a more rapid reduction in the rate of transpiration (Figure 3).

423

424 **4.4 | Na⁺ and K⁺ translocation to shoots by the xylem sap and K⁺/Na⁺ shoot homeostasis**

425 An apoplastic pathway strongly contributing to Na⁺ entry into the root and radial migration to
426 the root vasculature (the so-called bypass flow across the root to the xylem) has been
427 evidenced in rice in the presence of high Na⁺ concentrations (Faiyue, Al-Azzawi, & Flowers,
428 2012; Flam-Shepherd et al., 2018; Yeo, 1998). In our experimental conditions, the
429 concentration of Na⁺ in the xylem sap in both WT and *osk5.2* mutant plants was quite similar
430 to that in the hydroponic medium (50 mM) (Figure 4). Also, the xylem sap K⁺/Na⁺
431 concentration ratio decreased very rapidly down to values lower than 0.1 after one day of salt
432 treatment, and was then more than 10 times lower than the K⁺/Na⁺ root content ratio (Figure
433 S4). This indicates that the former ratio (in the xylem sap) was not likely to reflect the
434 corresponding ratio in the root symplasm. Altogether, these results support the hypothesis
435 that the bypass flow of Na⁺ was the major determinant of the migration of this cation towards
436 the xylem vasculature. In such conditions, the flux of Na⁺ translocated to the shoot becomes
437 proportional to the volumetric flow of xylem sap. Since the Na⁺ concentration of the xylem
438 sap was similar in the mutant and WT plants, the larger rate of transpiration under salt stress
439 in the mutant plants (Figure 3) due to impaired control of stomatal aperture is the major
440 determinant of the difference in Na⁺ translocation rate between the two types of plants
441 (Figure 5b). Thus, OsK5.2-dependent control of transpiration rate results in a reduction of

442 Na^+ translocation towards the shoots.

443 Despite the positive effect on salt stress tolerance of a reduction in xylem sap volumetric
444 flow, which decreases the flux of translocated Na^+ to shoots, a reduction in xylem sap
445 volumetric flow is also likely to have negative impact on the rate of K^+ translocation to
446 shoots (Figure 5a). Nevertheless, due to the dual role of *OsK5.2* in controlling transpiration
447 rate and K^+ secretion into xylem sap, WT plants expressing *OsK5.2* showed a smaller
448 reduction in the rate of K^+ translocation to shoots compared to mutant plants. In other words,
449 although *OsK5.2* activity in stomata decreases the transpiration rate (Figure 3) and thus has a
450 negative effect on K^+ translocation to shoots, the contribution of *OsK5.2* to K^+ loading into
451 the xylem sap (Figure 4a) outperforms the "negative" effect resulting from reduced xylem
452 volumetric flow (Figure 5a). This conclusion supports the hypothesis that the beneficial effect,
453 in terms of control of Na^+ translocation to shoots and tolerance to salinity, of the reduction in
454 transpiration rate upon salt stress is likely to integrate the plant ability to increase, or at least
455 maintain, the rate of K^+ secretion into the xylem sap.

456 Due to the beneficial effects of the overall *OsK5.2* activity, the ratio of the K^+ to Na^+
457 translocation rates towards the shoots (identical to the xylem sap K^+/Na^+ concentration ratio;
458 Figure 4c) is larger in WT than in *osk5.2* mutant plants. This is probably the main reason why
459 the kinetics of the decrease in shoot K^+/Na^+ content ratio is slower in WT than in mutant
460 plants (Figure 8b), and why the overall activity of *OsK5.2* contributes to salt tolerance
461 (Figure 1).

462

463 **4.5 | Contribution of a K^+ channel to plant salt tolerance**

464 Salt stress does not only strongly increase shoot Na^+ content but also leads to severe K^+
465 deficiency (Hauser & Horie, 2010; Marschner, 2011). Since insuring efficient root K^+ uptake
466 from soil appears as the primary way to insure shoot K^+ feeding, most studies aiming at
467 identifying salt tolerance determinants among K^+ transport systems have focused on root
468 uptake systems. Exposure to high salinity can substantially depolarize root periphery cells
469 and make passive K^+ uptake through inwardly rectifying K^+ channels thermodynamically
470 impossible (Rubio et al., 2020). High-affinity HAK/KUP/KT transporters, expected to rely on
471 pH gradients created by the H^+ -ATPase pump to energize inward K^+ fluxes through H^+ - K^+
472 symport mechanism, are therefore considered as the main K^+ transport systems taking part in
473 root K^+ uptake under high saline conditions. Several HAK transporters have been shown to
474 be involved in root K^+ uptake and thereby to contribute to plant salt tolerance: AtHAK5 in
475 Arabidopsis (Nieves-Cordones et al., 2010), and OsHAK1, OsHAK5, OsHAK16 and
476 OsHAK21 in rice (Chen et al., 2015; Feng et al., 2019; Shen et al., 2015; Yang et al., 2014).
477 KO mutations in these different genes have been shown to result in reduced K^+ uptake and
478 root K^+ content, and probably as a consequence, also in reduced K^+ translocation to shoots
479 and often reduced shoot K^+ contents. Such defects could be observed upon salt stress but also
480 in absence of saline treatment, and resulted in reduced plant growth in all conditions (Chen et
481 al., 2015; Feng et al., 2019; Nieves-Cordones et al., 2010; Shen et al., 2015; Yang et al.,
482 2014), Increased plant Na^+ uptake was noted in some mutants (Shen et al., 2015), which
483 could originate from higher root cell polarization (Nieves-Cordones et al., 2017). Also
484 supporting the importance of HAK-mediated plant K^+ uptake in salt tolerance, transcript level
485 variations in the *OsHAK1* gene between rice subspecies have been found to contribute to the

486 difference in their salt tolerance (Chen et al., 2015).

487 It is also well known that transport systems from the H⁺/cation antiporter families,
488 involved in K⁺ and Na⁺ intracellular compartmentalization, are major contributors to salt
489 tolerance (van Zelm et al., 2020) through their roles in Na⁺ compartmentalization and turgor
490 regulation, but also through indirect contributions to K⁺ homeostasis. For instance, increased
491 activity (due to overexpression in transgenic plants) of the antiporter AtNHX1 from
492 Arabidopsis or LeNHX2 from tomato has been shown to result in improved root K⁺ uptake
493 and higher K⁺ contents in all tissues. Such effects, which are beneficial to salt tolerance, have
494 been proposed to result from a decrease in cytosolic K⁺ concentration that these transport
495 systems would generate by compartmentalizing K⁺, which would lead to increased expression
496 and/or activity of high affinity K⁺ transporters involved in root K⁺ uptake (Leidi et al., 2010;
497 Huertas et al., 2013). Altogether, these studies provide evidence of strong interactions
498 between K⁺ uptake, compartmentalization and translocation to shoots.

499 Other K⁺ transport-mediated mechanisms of plant salt tolerance and in particular
500 mechanisms involving K⁺ channels, were reported but have not yet been deciphered.
501 Transcriptional regulation of a few K⁺ channel genes, especially the strong up-regulation of
502 the inward Shaker regulatory subunit *AtKCI* in leaves (Pilot et al., 2003), suggests a role of
503 inward K⁺ channels in salt tolerance, which has not been determined so far. Recently, reverse
504 genetics analyses suggested that the weak inwardly rectifying channel gene *OsAKT2*, which
505 is expressed in phloem, indirectly controls root Na⁺ uptake, thereby contributing to
506 maintenance of leaf K⁺/Na⁺ homeostasis upon salt stress (Tian et al., 2021).

507 Here, we showed that KO mutation in the outward Shaker K⁺ channel gene *OsK5.2* leads

508 to increased salt sensitivity. Lack of *OsK5.2* functional expression was found to result in
509 impaired growth in plants subjected to saline conditions but not in plants grown in standard
510 conditions (Figure 1), in contrast to what has been reported in KO mutant plants impaired in
511 root K^+ uptake, which mostly showed growth defects even in absence of salt stress (see
512 above). *OsK5.2* is involved in control of transpiration rate and in K^+ secretion into the xylem
513 sap, and these two functions together underlie its contribution to salt tolerance. It is also
514 worth to note that lack of *OsK5.2* activity results also in impaired net K^+ uptake under saline
515 conditions since both plant growth and whole plant K^+ contents were lower in *osk5.2* mutant
516 plants compared with the corresponding WT plants (Figure 1 and Figure 6). Together with
517 the defects in K^+ translocation to shoots under salt stress that have been reported in mutant
518 plants impaired in root K^+ uptake or in K^+ intracellular compartmentalization (see above), the
519 reduction in K^+ uptake resulting from lack of *OsK5.2* channel activity provides evidence that
520 the three functions, uptake, compartmentalization and translocation, are especially intensively
521 coordinated under saline conditions. In conclusion, the present results highlight
522 K^+ -channel-mediated mechanisms of salt tolerance, and provide a new possible target for
523 plant breeders towards the improvement of tolerance to salt stress in rice.

524

525 CONFLICT OF INTEREST

526 The authors declare no competing interests

527

528 AUTHOR CONTRIBUTIONS

529 A.-A.V., H.S., T.H.N. and J.Z. conceived the original research plans; A.-A.V., and D.T.L.

530 supervised the experiments; D.H., T.H.N. and J.Z. performed the experiments. A.-A.V., H.S.,
531 D.H., T.H.N. and J.Z. analyzed the data; A.-A.V., H.S. and J.Z. wrote the first draft of the
532 manuscript. All authors contributed to the article and approved the submitted version.

533

534 DATA AVAILABILITY STATEMENT

535 The data that support the findings of this study are available from the corresponding author
536 upon reasonable request

537

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544

545 **FIGURE LEGENDS**

546

547 **FIGURE 1** Effect of *OsK5.2* loss of function on rice plant phenotype in control and salt stress
548 conditions. Comparison of growth phenotype (a) and dry weight (b) between corresponding
549 wild-type and *osk5.2* mutant plants (black and white bars, respectively) issued from ASJA08
550 or ASHF06 lines (left and right panels, respectively) under control and salt treatment.
551 Six-week-old plants grown on hydroponic Yoshida medium were supplemented or not during

552 the last 7 days with 50 or 100 mM NaCl. Enlargements of leaf tips in 100 mM NaCl
553 treatment are shown in inserts at the right for the four genotypes. Scale bars = 10 cm in (a).
554 Means \pm SE, $n = 10$ in (b). Double stars above the bars denote statistically significant
555 differences between wild-type and *osk5.2* mutant plants (Student's t test, $P \leq 0.01$).

556

557 **FIGURE 2** Effect of saline conditions on *OsK5.2* transcript levels in roots and leaves.
558 Five-week-old rice plants cv Nipponbare hydroponically grown on Yoshida medium were
559 supplemented or not with 50 mM NaCl for 14 days. Salt-treated plants were thereafter
560 allowed to recover for 3 days on standard Yoshida medium. Expression data in roots (a) and
561 leaves (b) were determined by real-time quantitative RT-PCR. Means \pm SE ($n = 3$ biological
562 replicates under salt treatment after 1, 3, 7 and 14 days and recovery, and $n = 4$ under control
563 treatment, one plant in control condition sampled at each time of salt treatment). Different
564 letters indicate statistically significant differences (one-way ANOVA, Duncan's test, $P \leq$
565 0.05).

566

567 **FIGURE 3** Steady-state transpiration rates in wild-type and *osk5.2* mutant plants under
568 control and salt treatment conditions. Five-week-old plants hydroponically grown on Yoshida
569 medium were supplemented or not with 50 mM NaCl for 14 days. Left and right panels:
570 *osk5.2* mutant plants (\circ) issued from ASJA08 or ASHF06 lines, respectively, and the
571 corresponding wild-type plants (\bullet). Transpiration was measured after 1, 3, 7 and 14 days of
572 salt treatment (and at the same times for the plants maintained in control conditions). (a) and
573 (b): steady-state transpiration rates in light (panel a; ~ 3 h after light was switched on) and in

574 dark (panel b; ~5 h after light was switched off) conditions. Steady-state transpiration rate
575 was determined by dividing the average plant rate of water loss at steady-state (means of 3
576 values) by the total surface of the plant aerial parts. Means \pm SE; $n = 9$ under salt treatment
577 after 1, 3, 7, 14 days, and $n = 12$ under control conditions (3 plants in control condition
578 sampled at each time of salt treatment). Single and double stars denote statistically significant
579 differences between wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq$
580 0.01, respectively).

581

582 **FIGURE 4** Xylem sap K^+ and Na^+ concentrations in wild-type and *osk5.2* mutant plants
583 under control and salt treatment conditions. Five-week-old plants hydroponically grown on
584 Yoshida medium were supplemented or not with 50 mM NaCl for 14 days. Left and right
585 panels: *osk5.2* mutant plants (\circ) issued from ASJA08 or ASHF06 lines, respectively, and the
586 corresponding wild-type plants (\bullet). Xylem sap exudates were collected after 1, 3, 7 and 14
587 days of salt treatment (and at the same times for the plants maintained in control conditions).
588 (a) and (b): K^+ (a) and Na^+ (b) concentrations assayed in the collected xylem sap samples. (c)
589 K^+/Na^+ concentration ratios deduced from (a) and (b). Means \pm SE; $n = 9$ under salt treatment
590 after 1, 3, 7, 14 days, and $n = 12$ under control conditions (3 plants in control condition
591 sampled at each time of salt treatment). Single and double stars denote statistically significant
592 differences between wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq$
593 0.01, respectively).

594

595 **FIGURE 5** K^+ and Na^+ fluxes arriving at light in leaves of wild-type and *osk5.2* mutant plants

596 under control and salt treatment conditions. (a) and (b): normalized values (expressed per leaf
597 surface) of K^+ (a) and Na^+ (b) fluxes assessed by multiplying the mean transpiration rates
598 (data from Figure 3) by the corresponding K^+ or Na^+ concentrations in xylem sap (data from
599 Figure 4). Left and right panels: *osk5.2* mutant plants (\circ) and corresponding wild-type plants
600 (\bullet) issued from ASJA08 (left) or ASHF06 (right) lines. Means \pm SE; $n = 9$ under salt
601 treatment after 1, 3, 7 and 14 days, and $n = 12$ under control conditions. See legends to
602 Figures 3 and 4. Single and double stars denote statistically significant differences between
603 the wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

604

605 **FIGURE 6** Root and Shoot K^+ contents in wild-type and *osk5.2* mutant plants under control
606 and salt treatment conditions. Five-week-old plants hydroponically grown on Yoshida
607 medium were supplemented or not with 50 mM NaCl for 14 days. Left and right panels:
608 *osk5.2* mutant plants (\circ) and the corresponding wild-type plants (\bullet) issued from ASJA08
609 (left) or ASHF06 (right) lines. Roots and shoots were sampled after 1, 3, 7 and 14 days of salt
610 treatment (and at the same times for the plants maintained in control conditions). (a), (b) and
611 (c): K^+ contents in roots, shoots and whole plant, respectively. Means \pm SE; $n = 15$ to 20 for
612 the wild-type and mutant plants issued from the ASJA08 line, and 9 to 12 for wild-type and
613 mutant plants issued from the ASHF06 line. Single and double stars denote statistically
614 significant differences between the wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq$
615 0.05 and $P \leq 0.01$, respectively).

616

617 **FIGURE 7** Root and Shoot Na^+ contents in wild-type and *osk5.2* mutant plants under control

618 and salt treatment conditions. Same plants as in Figure 6. (a), (b) and (c): Na⁺ contents in
619 roots, shoots and whole plant, respectively. Means \pm SE; $n = 15$ to 20 for the wild-type and
620 mutant plants issued from the ASJA08 line, and 9 to 12 for wild-type and mutant plants
621 issued from the ASHF06 line. Single and double stars denote statistically significant
622 differences between the wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq$
623 0.01, respectively).

624

625 **FIGURE 8** Root and shoot K⁺/Na⁺ content ratio in wild-type and *osk5.2* mutant plants under
626 control and salt treatment conditions. Same experiment as in Figures 6 and 7. K⁺/Na⁺ content
627 ratio: K⁺ content from Figure 6 divided by the corresponding Na⁺ content from Figure 7. (a)
628 and (b): K⁺/Na⁺ content ratio in roots and shoots. Left and right panels: *osk5.2* mutant plants
629 (○) and corresponding wild-type plants (●) issued from ASJA08 (left) or ASHF06 (right)
630 lines. Means \pm SE; $n = 15$ to 20 for the wild-type and mutant plants issued from the ASJA08
631 line, and 9 to 12 for wild-type and mutant plants issued from the ASHF06 line. Single and
632 double stars denote statistically significant differences between the wild-type and *osk5.2*
633 mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

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778 SUPPORTING INFORMATION

779 Additional supporting information may be found online in the Supporting Information section
780 at the end of this article.

781

782 **Table S1:** Primers used for qRT-PCR experiments

783 **Figure S1** Effect of salt treatment on shoot and root length of wild-type (black bars) and
784 *osk5.2* mutant (white bars) plants issued from ASJA08 (top) or ASHF06 (bottom) lines.

785 **Figure S2** Shoot and root water contents of wild-type and *osk5.2* mutant plants under control
786 and salt treatment conditions.

787 **FIGURE S3** Scanning electron micrographs showing stomatal shape on the abaxial flag leaf
788 side from *osk5.2* mutant (right column) and corresponding wild-type (left column) plants
789 issued from ASJA08 (top) or ASHF06 (bottom) lines.

790 **Figure S4** The ionic composition of the xylem sap does not reflect the K⁺ and Na⁺ relative
791 contents of the roots.

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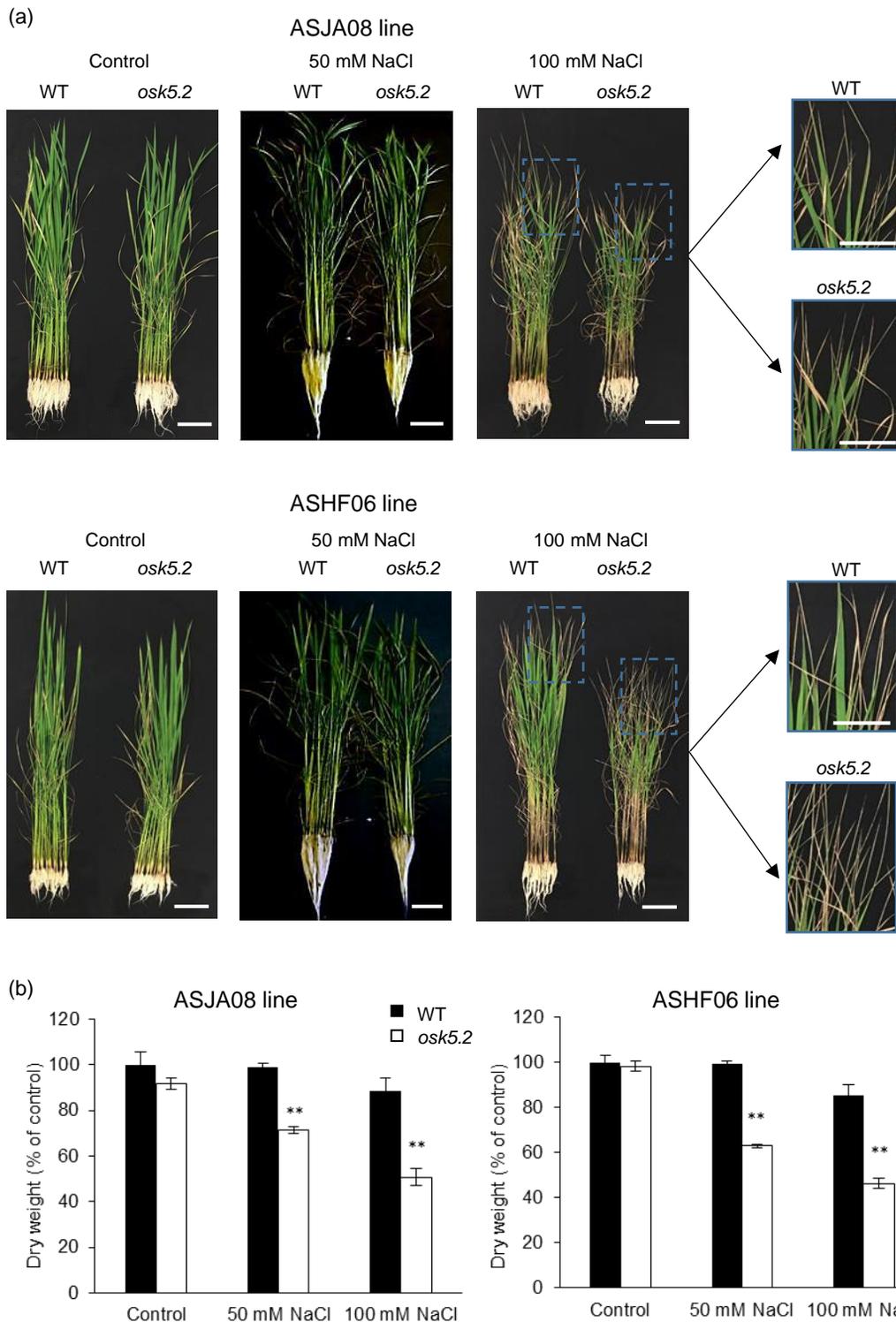


FIGURE 1 Effect of *Osk5.2* loss of function on rice plant phenotype in control and salt stress conditions. Comparison of growth phenotype (a) and dry weight (b) between corresponding wild-type and *osk5.2* mutant plants (black and white bars, respectively) issued from ASJA08 or ASHF06 lines (left and right panels, respectively) under control and salt treatment. Six-week-old plants grown on hydroponic Yoshida medium were supplemented or not during the last 7 days with 50 or 100 mM NaCl. Enlargements of leaf tips in 100 mM NaCl treatment are shown in inserts at the right for the four genotypes. Scale bars = 10 cm in (a). Means \pm SE, $n = 10$ in (b). Double stars above the bars denote statistically significant differences between wild-type and *osk5.2* mutant plants (Student's t test, $P \leq 0.01$).

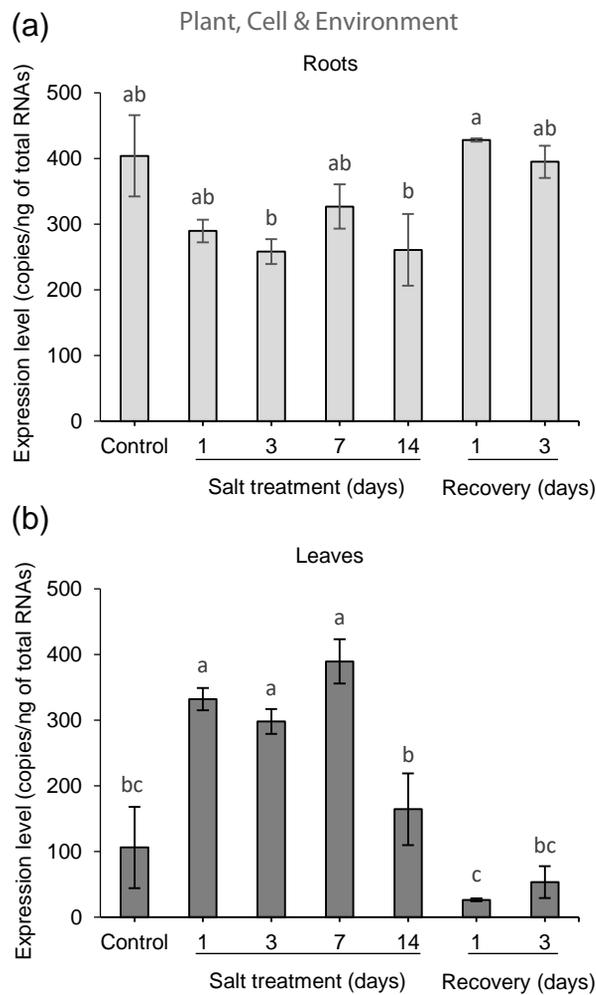


FIGURE 2 Effect of saline conditions on *OsK5.2* transcript levels in roots and leaves. Five-week-old rice plants cv Nipponbare hydroponically grown on Yoshida medium were supplemented or not with 50 mM NaCl for 14 days. Salt-treated plants were thereafter allowed to recover for 3 days on standard Yoshida medium. Expression data in roots (a) and leaves (b) were determined by real-time quantitative RT-PCR. Means \pm SE ($n = 3$ biological replicates under salt treatment after 1, 3, 7 and 14 days and recovery, and $n = 4$ under control treatment sampled at each time of salt treatment). Different letters indicate statistically significant differences (One-way ANOVA, Duncan's test, $P \leq 0.05$).

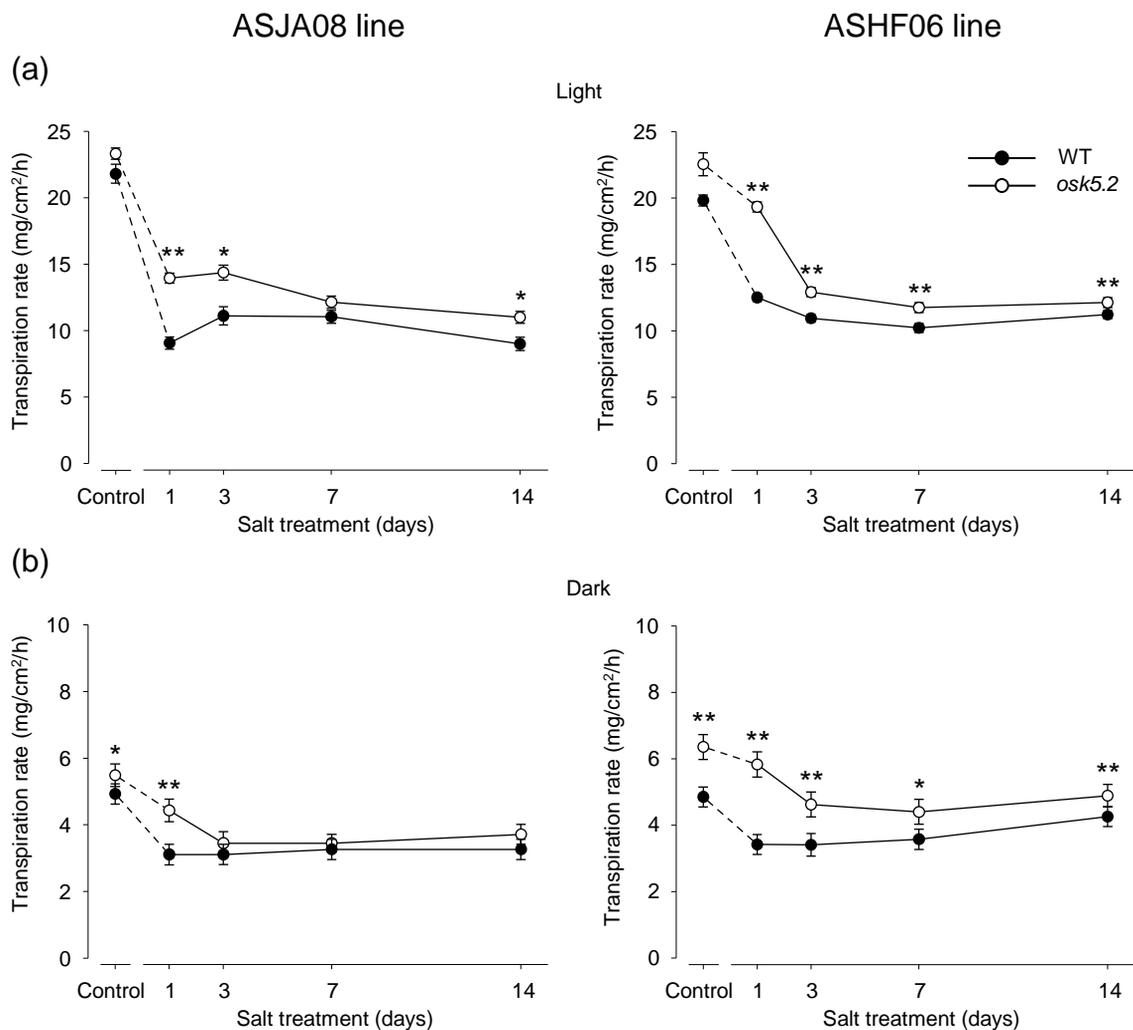


FIGURE 3 Steady-state transpiration rates in wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Five-week-old plants hydroponically grown on Yoshida medium were supplemented or not with 50 mM NaCl for 14 days. Left and right panels: *osk5.2* mutant plants (○) issued from ASJA08 or ASHF06 lines, respectively, and the corresponding wild-type plants (●). Transpiration was measured after 1, 3, 7 and 14 days of salt treatment (and at the same times for the plants maintained in control conditions). (a) and (b): steady-state transpiration rates in light (panel a; ~3 h after light was switched on) and in dark (panel b; ~5 h after light was switched off) conditions. Steady-state transpiration rate was determined by dividing the average plant rate of water loss at steady-state (means of 3 values) by the total surface of the plant aerial parts. Means \pm SE; $n = 9$ under salt treatment after 1, 3, 7, 14 days, and $n = 12$ under control conditions (3 plants in control condition sampled at each time of salt treatment). Single and double stars denote statistically significant differences between wild-type and *osk5.2* mutant plants (Student's *t* test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

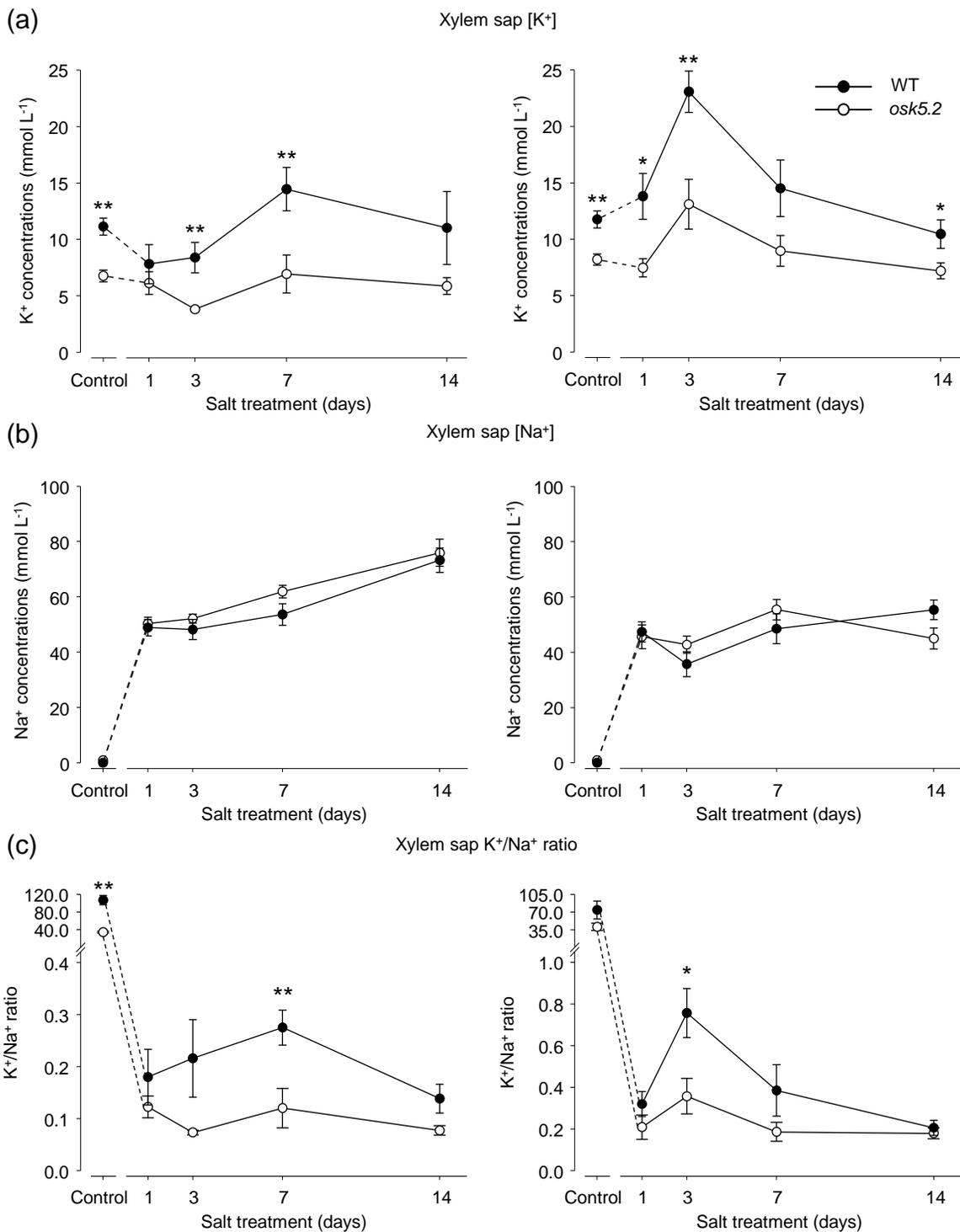


FIGURE 4 Xylem sap K⁺ and Na⁺ concentrations in wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Five-week-old plants hydroponically grown on Yoshida medium were supplemented or not with 50 mM NaCl for 14 days. Left and right panels: *osk5.2* mutant plants (○) issued from ASJA08 or ASHF06 lines, respectively, and the corresponding wild-type plants (●). Xylem sap exudates were collected after 1, 3, 7 and 14 days of salt treatment (and at the same times for the plants maintained in control conditions). (a) and (b): K⁺ (a) and Na⁺ (b) concentrations assayed in the collected xylem sap samples. (c) K⁺/Na⁺ concentration ratios deduced from (a) and (b). Means ± SE; *n* = 9 under salt treatment after 1, 3, 7, 14 days, and *n* = 12 under control conditions (3 plants in control condition sampled at each time of salt treatment). Single and double stars denote statistically significant differences between wild-type and *osk5.2* mutant plants (Student's *t* test, *P* ≤ 0.05 and *P* ≤ 0.01, respectively).

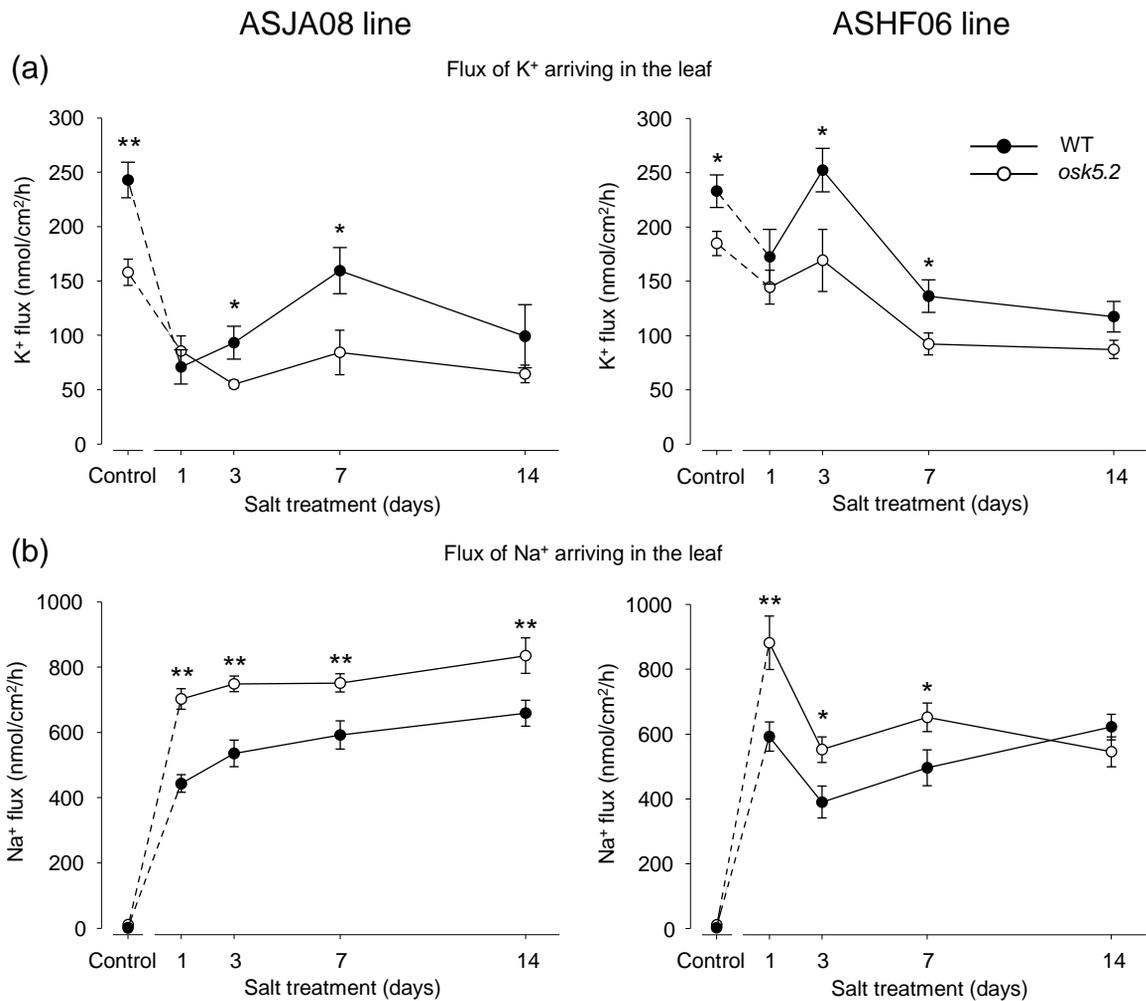


FIGURE 5 K⁺ and Na⁺ fluxes arriving at light in leaves of wild-type and *osk5.2* mutant plants under control and salt treatment conditions. (a) and (b): normalized values (expressed per leaf surface) of K⁺ (a) and Na⁺ (b) fluxes assessed by multiplying the mean transpiration rates (data from Figure 3) by the corresponding K⁺ or Na⁺ concentrations in xylem sap (data from Figure 4). Left and right panels: *osk5.2* mutant plants (○) and corresponding wild-type plants (●) issued from ASJA08 (left) or ASHF06 (right) lines. Means ± SE; $n = 9$ under salt treatment after 1, 3, 7 and 14 days, and $n = 12$ under control conditions. See legends to Figures 3 and 4. Single and double stars denote statistically significant differences between the wild-type and *osk5.2* mutant plants (Student's t test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

ASJA08 line

ASHF06 line

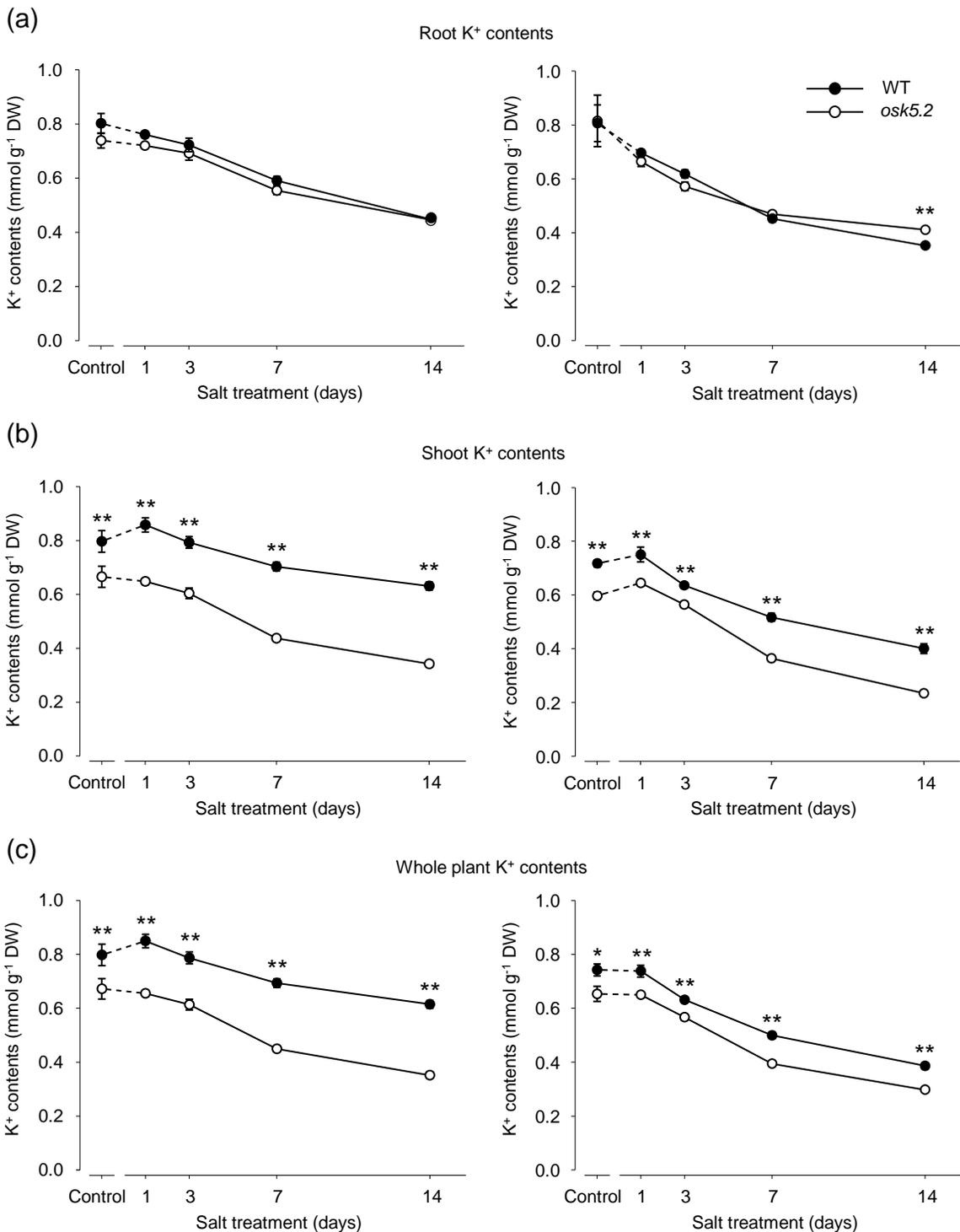


FIGURE 6 Root and Shoot K⁺ contents in wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Five-week-old plants hydroponically grown on Yoshida medium were supplemented or not with 50 mM NaCl for 14 days. Left and right panels: *osk5.2* mutant plants (○) and the corresponding wild-type plants (●) issued from ASJA08 (left) or ASHF06 (right) lines. Roots and shoots were sampled after 1, 3, 7 and 14 days of salt treatment (and at the same times for the plants maintained in control conditions). (a), (b) and (c): K⁺ contents in roots, shoots and whole plant, respectively. Means ± SE; *n* = 15 to 20 for the wild-type and mutant plants issued from the ASJA08 line, and 9 to 12 for wild-type and mutant plants issued from the ASHF06 line. Single and double stars denote statistically significant differences between the wild-type and *osk5.2* mutant plants (Student's *t* test, *P* ≤ 0.05 and *P* ≤ 0.01, respectively).

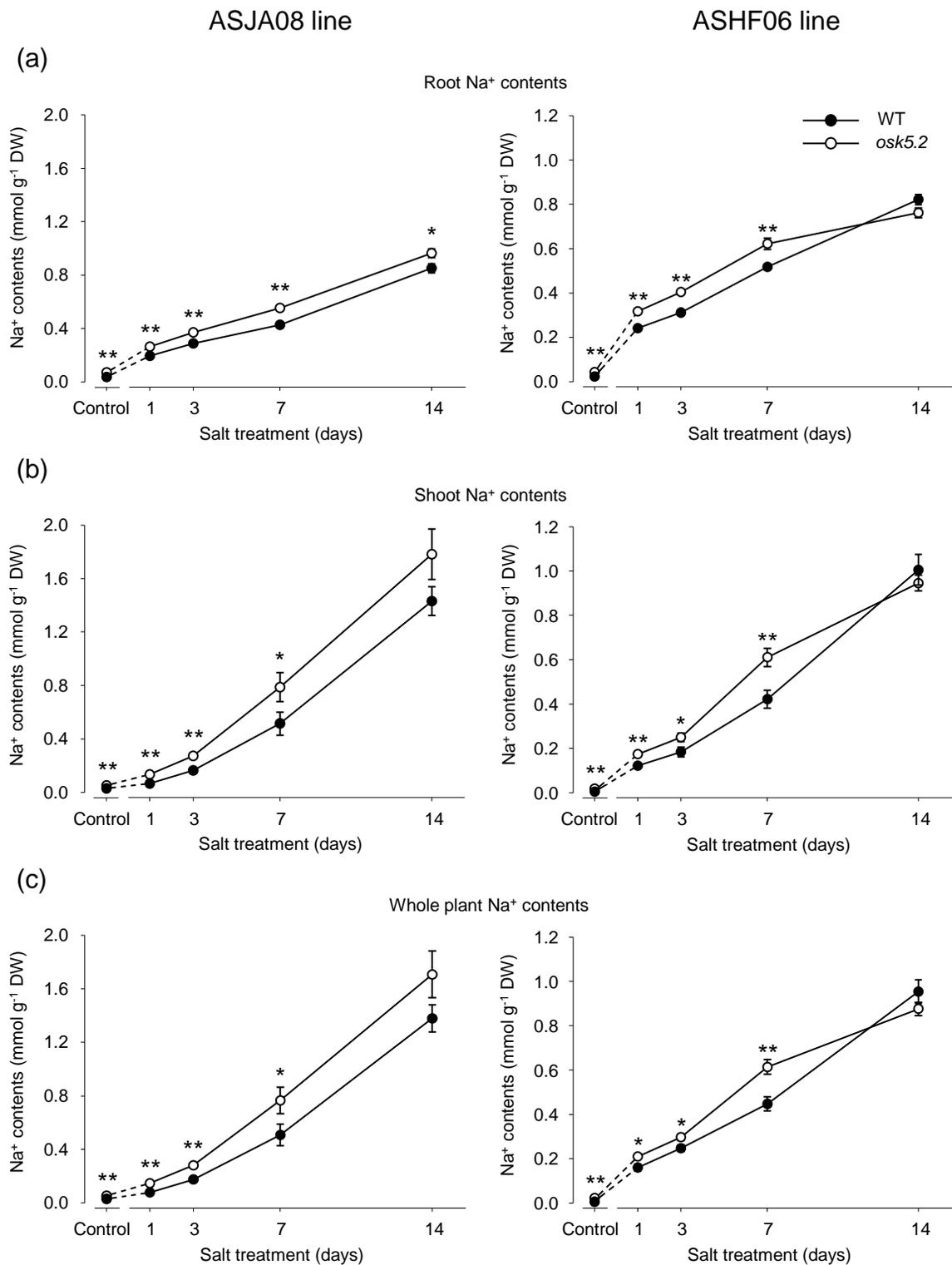


FIGURE 7 Root and Shoot Na⁺ contents in wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Same plants as in Figure 6. (a), (b) and (c): Na⁺ contents in roots, shoots and whole plant, respectively. Means \pm SE; $n = 15$ to 20 for the wild-type and mutant plants issued from the ASJA08 line, and 9 to 12 for wild-type and mutant plants issued from the ASHF06 line. Single and double stars denote statistically significant differences between the wild-type and *osk5.2* mutant plants (Student's t test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

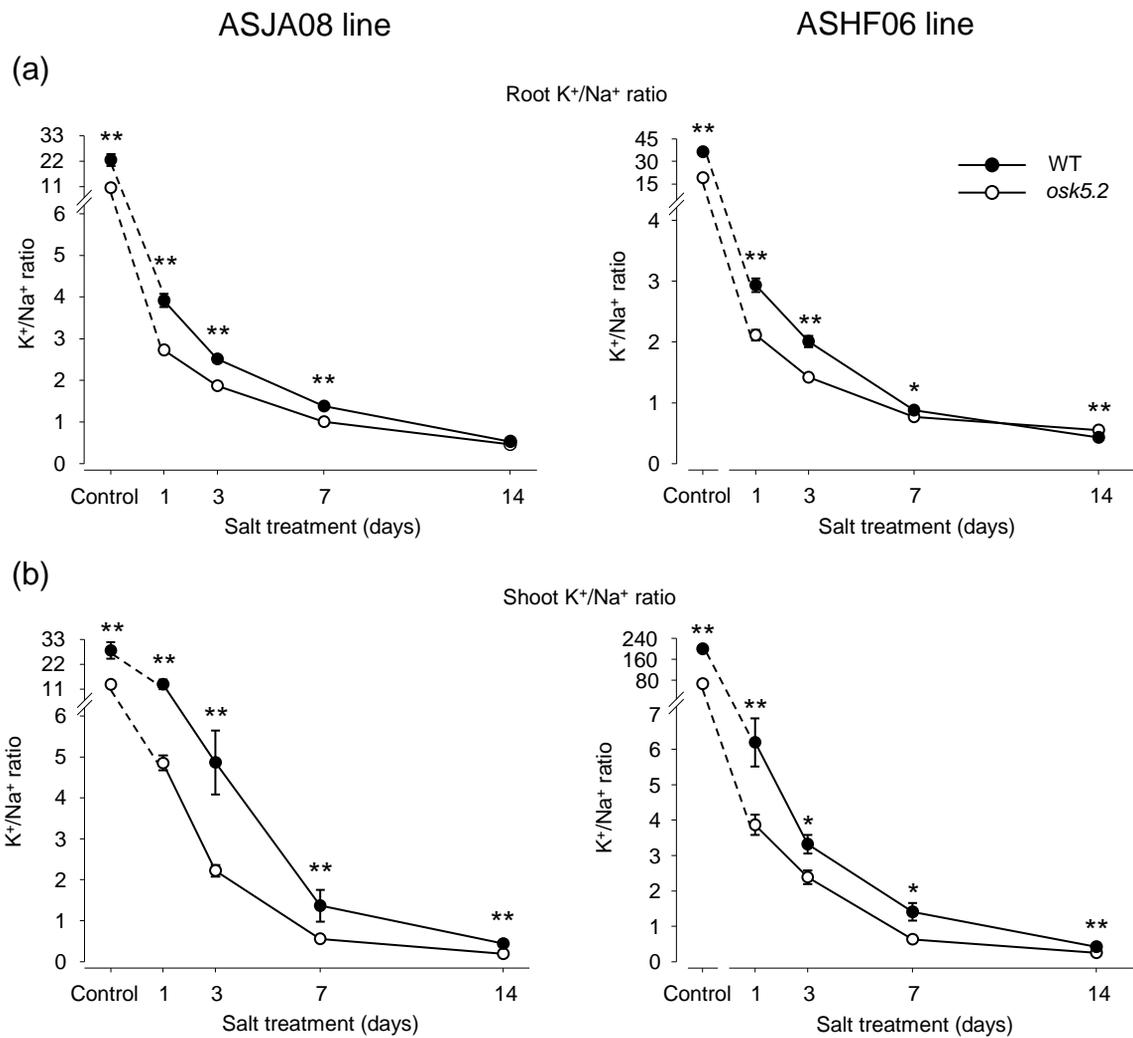


FIGURE 8 Root and shoot K^+/Na^+ content ratio in wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Same experiment as in Figures 6 and 7. K^+/Na^+ content ratio: K^+ content from Figure 6 divided by the corresponding Na^+ content from Figure 7. (a) and (b): K^+/Na^+ content ratio in roots and shoots. Left and right panels: *osk5.2* mutant plants (\circ) and corresponding wild-type plants (\bullet) issued from ASJA08 (left) or ASHF06 (right) lines. Means \pm SE; $n = 15$ to 20 for the wild-type and mutant plants issued from the ASJA08 line, and 9 to 12 for wild-type and mutant plants issued from the ASHF06 line. Single and double stars denote statistically significant differences between the wild-type and *osk5.2* mutant plants (Student's t test, $P \leq 0.05$ and $P \leq 0.01$, respectively).

Table S1: Primers used for qRT-PCR experiments.

Gene	Primer name	Sequence (5'-3')	Amplicon length (bp)
<i>OsK5.2</i>	qPCR-OsK52-F358	TTTGGCTTCTTCAGGGGGCT	470 *
	qPCR-OsK52-R808	TCTCCCAACTGCAAGCTCCC	
	qPCR-OsK52-F467	CTGACACCTACCGCATGGTT	D-164
	qPCR-OsK52-R611	TCGTGTCAACCGAATCCACA	
<i>SMT3</i>	SMT3B303F	GGGAGGAGGACAAGAAGC	273 *
	SMT3B303R	CTCCAGTCTGGTGGAGCAT	
	QRT94SMT3-F	CCTCAAGGTCAAGGGACAGG	D-94
	QRT94SMT3-R	ACGGTCACAATAGGCGTTCA	
<i>Tip41</i>	Tip41B389F	TGGTTTTTGGGGAGAGTTTC	389 *
	Tip41B389R	TGAAATGCCATTATCGGCTA	
	QRT101Tip41-F	TGGGAGTGATGCTTTGGTTC	D-101
	QRT101Tip41-R	CAAGGTCAATCCGATCCTCA	
<i>eEF-1-beta2</i>	eEFB2Pcs	TGGTGAGGAGACTGAAGAGG	418 *
	eEFB2Pcas	CTTCCGGATTTTTCTTTTTATC	
	qPCR-eEF1b2-F413	TGGGAAGTCCTCAGTGTTGC	D-136
	qPCR-eEF1b2-R549	GTAACCAACCGGGACAAGCT	

“D”, pair of primers for transcript level quantification, that anneal on cDNA fragments derived from two exons. “*” refers to big amplicons that were used as a template for the amplification of calibration standards.

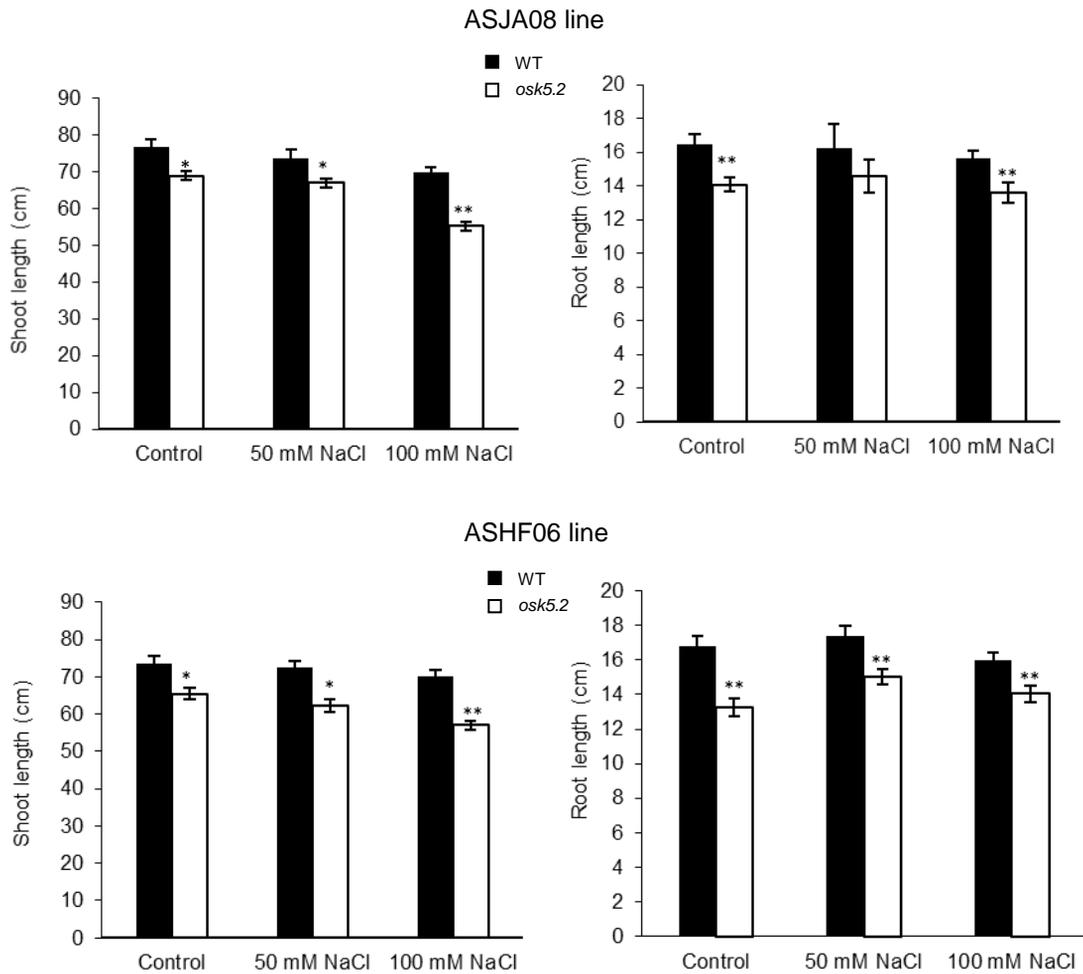


Figure S1 Effect of salt treatment on shoot and root length of wild-type (black bars) and *osk5.2* mutant (white bars) plants issued from ASJA08 (top) or ASHF06 (bottom) lines. Five-week-old rice plants hydroponically grown on Yoshida medium were supplemented or not with 50 or 100 mM NaCl for 7 days. Means \pm SE ($n = 10$ plants). One or 2 stars above bars corresponding to mutant plants indicate statistically significant differences with respect to the corresponding wild-type plants (Student's t test, $P < 0.05$ and < 0.01 , respectively).

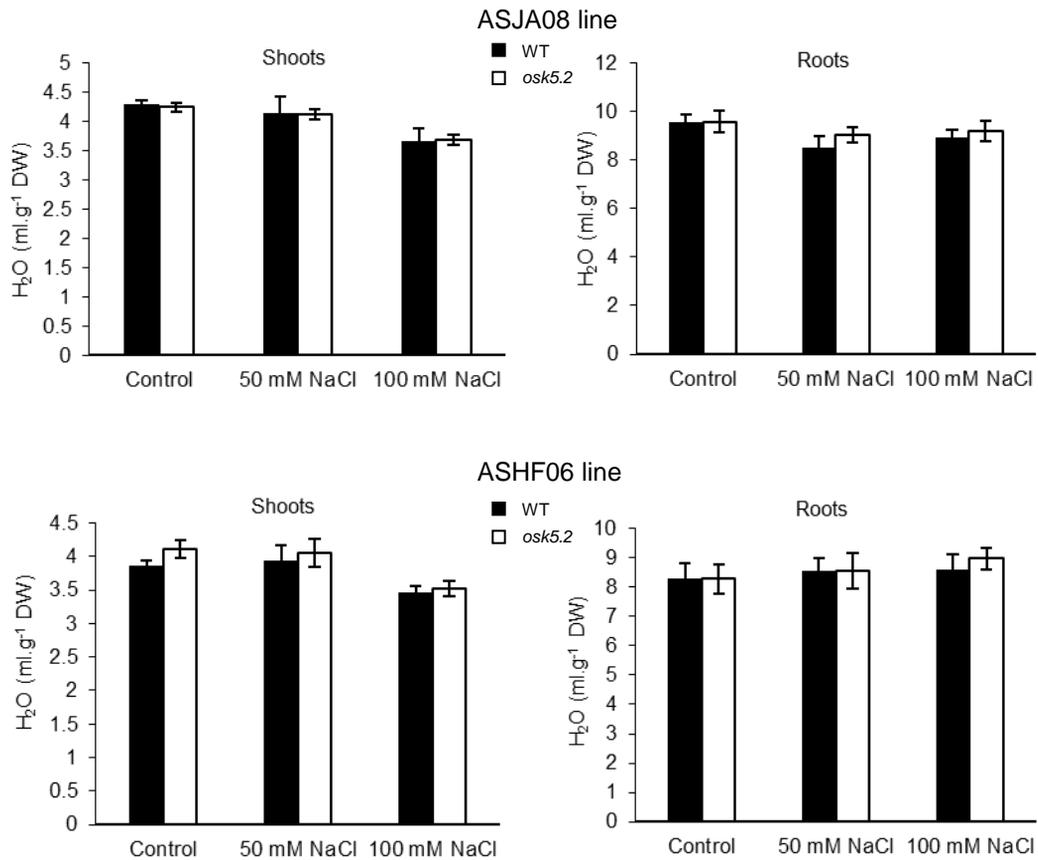


Figure S2 Shoot and root water contents of wild-type and *osk5.2* mutant plants under control and salt treatment conditions. Five-week-old rice plants hydroponically grown on Yoshida medium were supplemented or not with 50 or 100 mM NaCl for 7 days. Top and bottom panels: *osk5.2* mutant plants (white bars) and corresponding wild-type plants (black bars) issued from ASJA08 (top) or ASHF06 (bottom) lines. Means \pm SE ($n = 10$). Absence of star above the bars indicates no statistically significant difference between the wild-type and *osk5.2* mutant plants (Student's t test, $P > 0.05$).

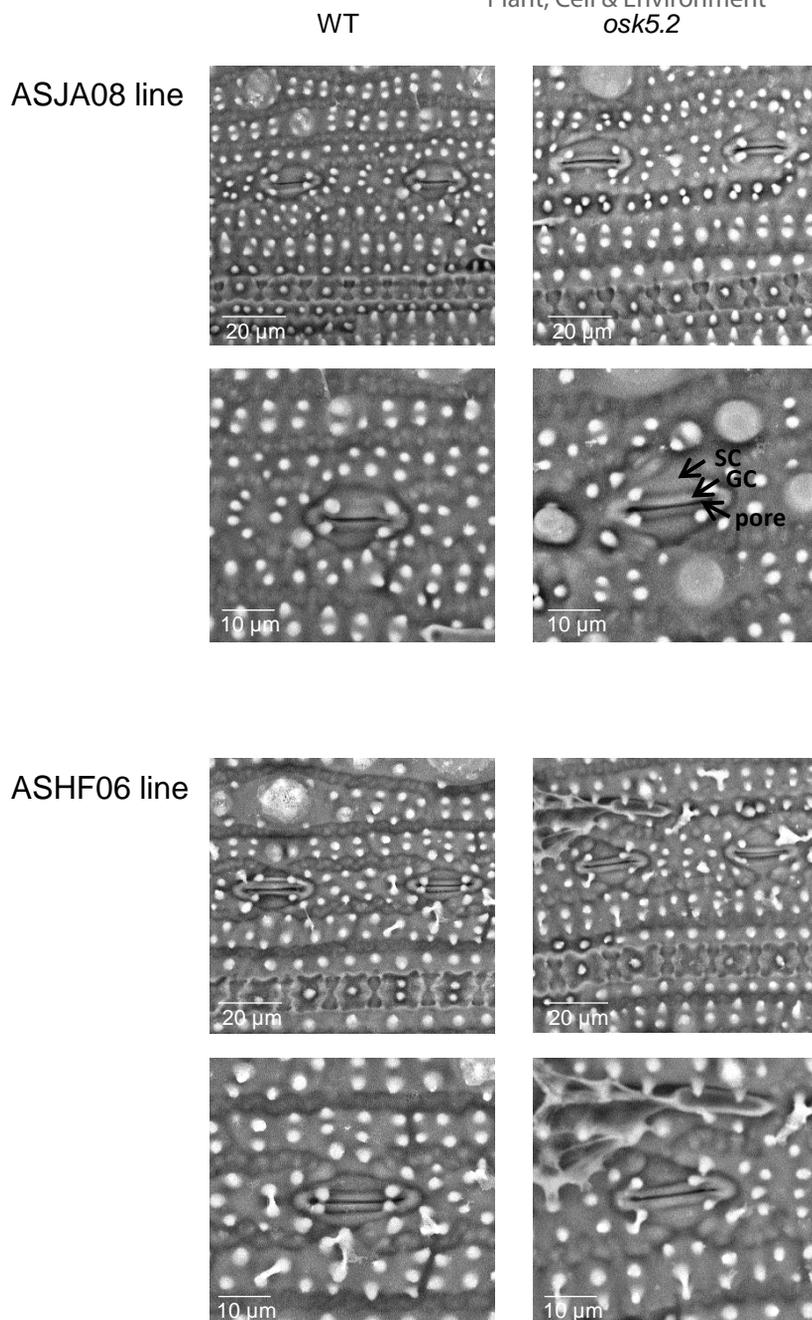


FIGURE S3 Scanning electron micrographs showing stomatal shape on the abaxial flag leaf side from *osk5.2* mutant (right column) and corresponding wild-type (left column) plants issued from ASJA08 (top) or ASHF06 (bottom) lines. Plants were grown in control conditions (Yoshida hydroponic medium). A ca. 1 cm-length portion of leaf, cut from an illuminated plant, was stuck on a thin layer of OCT cryo-embedding matrix (Carlroth) and quickly frozen at -14°C . Observations were performed on 30 stomata from 3 plants per genotype using a benchtop Phenom Pro X scanning electron microscope (Phenom World, Eindhoven, The Netherlands) using a backscattered electron detector with an acceleration voltage of 15 kV in image mode. GC: guard cell; SC: subsidiary cell.

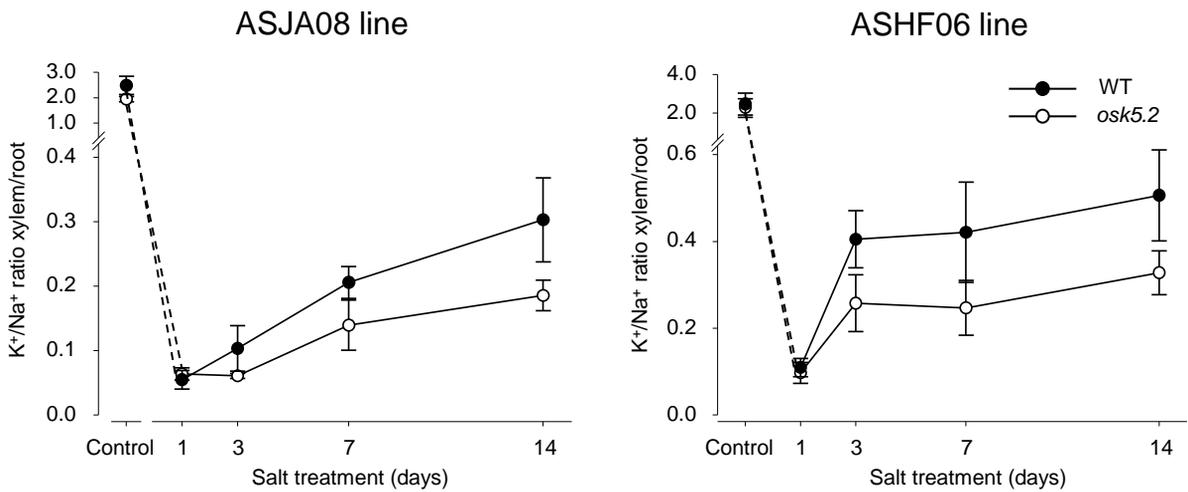


FIGURE S4 The ionic composition of the xylem sap does not reflect the K^+ and Na^+ relative contents of the roots. Plants were subjected to a saline treatments (50 mM NaCl for 2 weeks; x axis). Y axis: xylem sap K^+/Na^+ concentration ratio (data from Figure 4c) reported to the root K^+/Na^+ content ratio (data from Figure 8a). Left and right panels: *osk5.2* mutant plants (○) and corresponding wild-type plants (●) issued from ASJA08 (left) or ASHF06 (right) lines. Means \pm SE; $n = 9$ under salt treatment after 1, 3, 7 and 14 days, and $n = 12$ under control conditions. See legends to Figures 4 and 8.