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1 **Short Title:** Rice Salt Overly Sensitive 1

2

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6

7 **A Critical Role of Sodium Flux via the Plasma Membrane Na⁺/H⁺**

8 **Exchanger SOS1 in the Salt Tolerance of Rice**

9

10

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38 **Author's Contributions**

39

40 HEM, AL, JPH, IV, FGA, JE, JLF, IM, DM, EOL and FJQ performed the experimental work;
41 EL, JPH, IV and JMP did the transcriptomic analyses; IV, SYL, DJY, EG, MA, EOL, JMP,
42 and FJQ designed the research; all authors contributed to the discussion and
43 interpretation of results; JPH, JMP and FJQ wrote the article; IV, MB, EG, and EOL
44 revised and edited the manuscript.

45

46 **One-sentence Summary**

47

48 The plasma membrane Na^+/H^+ exchanger SOS1 plays a major role in the salt tolerance
49 of rice by controlling Na^+ uptake and root-shoot partitioning.

50

51 **ABSTRACT**

52

53 Rice (*Oryza sativa*) stands among the world's most important crop species. Rice is salt-
54 sensitive and the undue accumulation of sodium ions (Na^+) in shoots has the strongest
55 negative correlation with rice productivity under long-term salinity. The plasma
56 membrane Na^+/H^+ exchanger protein SOS1 is the sole Na^+ efflux transporter that has
57 been genetically characterized to date. Here, the importance of SOS1-facilitated Na^+
58 flux in the salt tolerance of rice was analyzed in a reverse-genetics approach. A *sos1*
59 loss-of-function mutant displayed exceptional salt sensitivity that was correlated with
60 excessive Na^+ intake and impaired Na^+ loading into the xylem, thus indicating that
61 SOS1 controls net root Na^+ uptake and long-distance Na^+ transport to shoots. The
62 acute Na^+ sensitivity of *sos1* plants at low NaCl concentrations allowed analysis of the
63 transcriptional response to sodicity stress without effects of the osmotic stress
64 intrinsic to high-salinity treatments. In contrast to that in the wild type, *sos1* mutant
65 roots displayed preferential down-regulation of stress-related genes in response to salt
66 treatment, despite the greater intensity of stress experienced by the mutant. These
67 results suggest there is impaired stress detection or an inability to mount a
68 comprehensive response to salinity in *sos1*. In summary, the plasma membrane Na^+/H^+
69 exchanger SOS1 plays a major role in the salt tolerance of rice by controlling Na^+
70 homeostasis and possibly contributing to the sensing of sodicity stress.

71

72 **KEYWORDS**

73

74 Rice, sodium transport, SOS1, salinity signaling

75

76

77 **INTRODUCTION**

78

79 Rice (*Oryza sativa*) is the most important salt-sensitive cereal crop (Hoang et al., 2016).
80 Rice is grown in a diverse range of environments with varying soil-water conditions.
81 Salinity is an intrinsic problem in coastal areas and river deltas where rice farming
82 predominates, but is also a worsening problem in inland areas due to the buildup of
83 secondary salinization of the soil surface as a consequence of excessive use of
84 irrigation with poor quality water and insufficient drainage (Thomson et al., 2010).
85 Excessive amounts of salts in the soil solution cause substantial declines in the
86 productivity of many agricultural plants, and in rice as little as 3.5 dS/m (2.1 g/L of
87 salts) is sufficient to curtail 10% of yield, and 7.2 dS/m (4.3 g/L) producing 50% yield
88 loss (Munns and Tester, 2008; Hoang et al., 2016).

89

90 Salinity poses two main challenges to plants. One is osmotic stress due to excess
91 solutes outside the roots that reduce the ability of plants to extract soil water, which
92 affects cell turgor and expansion immediately. Secondly, the ionic stress that is most
93 often caused by excessive influx of Na⁺ ions into the plant leads to interruption of
94 metabolic processes (Hasegawa et al., 2000; Munns and Tester, 2008). Physiological
95 and genetic studies indicate that a range of traits, such as low shoot Na⁺ content,
96 preservation of the K⁺ status of the whole plant, vacuolar compartmentation of salts in
97 older leaves, intrinsic cellular tolerance, and plant vigor, could incrementally improve
98 the ability of rice plants to cope with salinity (Yeo et al., 1990; Thomson et al., 2010).
99 Integration of all these physiological mechanisms for salinity tolerance by pyramiding
100 superior alleles controlling different processes is the long-term goal to increase the
101 level of salt tolerance in rice (Gregorio et al., 2002).

102

103 Sustained plant growth under salt stress requires tight control of Na⁺ uptake, Na⁺
104 redistribution among organs by long-distance transport, and Na⁺ accumulation into cell
105 vacuoles for detoxification and osmotic balance (Munns and Tester, 2008). The ability
106 to manage Na⁺ content has partly a cellular basis since rice protoplasts derived from
107 cultivars with contrasting salt tolerance showed that halotolerance depended on
108 reduced Na⁺ uptake and a fast compartmentation of Na⁺ into vacuoles (Kader and

109 Lindberg, 2005), whereas enhancement of Na⁺ efflux yielded salt-tolerant calli (Zhu
110 and Wu, 2008). At the whole-plant level, comparative studies have shown that salinity-
111 tolerant rice cultivars accumulate less Na⁺ in leaves and shoots compared to that in
112 salinity-sensitive rice cultivars (Golldack et al., 2003; Lee et al., 2003; Lin et al., 2004;
113 Ren et al., 2005), and that shoot Na⁺ accumulation had the strongest negative
114 correlation with the performance of contrasting rice genotypes under long-term
115 salinity (Coskun et al., 2013). Hence, the quantitative measure of the Na⁺ and K⁺
116 concentrations in the shoot in salinized conditions has been often employed as the
117 trait screened in quantitative trait loci (QTL) mapping and breeding programs (Hoang
118 et al., 2016). Known proteins governing the partitioning of Na⁺ between roots and
119 shoots are members of the so-called High-affinity Potassium Transporters (HKT), albeit
120 they have repeatedly been shown to behave as Na⁺ transporters mediating either
121 channel-like Na⁺ uniport (class I or HKT1 group) or Na⁺-K⁺ symport (class II or HKT2
122 group). Generally, dicot species have only a few *HKT* genes in their genomes preferably
123 encoding class-I HKT proteins, whereas monocots have multiple *HKT* genes of both
124 classes. The rice protein OsHKT1;5/SCK1 and the wheat proteins TmHKT1;4/ and
125 TmHKT1;5, all of them class-I proteins, mediate the retrieval of Na⁺ from the xylem
126 sap, thereby restricting the transfer of Na⁺ to shoots. Rice OsHKT1;4 also restricts leaf
127 sheath-to-blade Na⁺ transfer under salinity stress (Cotsaftis et al., 2012; Suzuki et al.,
128 2016).

129

130 Although several candidate proteins have been suggested, no major plasma
131 membrane transporters responsible for the bulk influx of Na⁺ into root cells have as
132 yet been conclusively identified at the genetic level and it seems likely that multiple
133 transport proteins and systems incrementally contribute to Na⁺ influx (Kronzucker and
134 Britto, 2011; Nieves-Cordones et al., 2016). In rice, the HKT2;1 protein that is
135 expressed in the root cortex and endodermis may provide an entry pathway for
136 'nutritional' Na⁺ uptake under K⁺ limitation to support cell expansion and plant growth
137 (Garcia-deblas et al., 2003; Horie et al., 2007). Sodium may also leak into the root stele
138 of rice plants through anatomical discontinuities in the endodermis produced by the
139 emergence of secondary roots (Yeo et al., 1987), or through the lateral roots
140 themselves (Faiyue et al., 2010). For Na⁺ transport in the opposite (efflux) direction,

141 only the SOS1 transporter (Salt Overly-Sensitive 1) has been genetically characterized
142 (Shi et al., 2002; Oh et al., 2009; Olias et al., 2009). In dicots, SOS1 controls net Na⁺
143 uptake by roots and long-distance transport to shoots (Oh et al., 2009; Olias et al.,
144 2009). Thus, the opposing yet concerted activity of SOS1 in xylem loading and of HKT1-
145 like proteins in xylem unloading might determine the amount of Na⁺ that is eventually
146 exported from roots to shoots. Although HKT proteins of cereal crops is well
147 established to play a major role in their salt tolerance, the contribution of Na⁺ loading
148 into the xylem by specific transporters remains to be determined. Likewise, the
149 regulatory cross-talk of these complementary transport systems has not been explored
150 (Ji et al., 2013).

151

152 Little is known about the regulation of HKT proteins. *HKT* gene expression is often
153 modulated by K⁺ availability and soil salinity, but the post-transcriptional regulation of
154 HKT transporters remains entirely unknown. In rice, the Na⁺ uptake gene *HKT2;1* is
155 rapidly repressed in roots by as little as 30 mM NaCl to curtail Na⁺ entry (Horie et al.,
156 2007). Surprisingly, *HKT1;4* expression is also down-regulated proportionally to the
157 severity of salinity treatment during the vegetative growth stage, and RNAi plants
158 showed no differences in salt sensitivity or in Na⁺ content (Suzuki et al., 2016). A
159 contribution of *HKT1;4* towards the prevention of Na⁺ accumulation in flag leaf blades
160 was found in the reproductive stage only (Suzuki et al., 2016). *OsHKT1;1*, involved in
161 Na⁺ re-circulation, is expressed mainly in the phloem of leaf blades and up-regulated
162 by salt stress (Wang et al., 2015). The transcription factor OsMYBc binds to the
163 *OsHKT1;1* promoter and loss of OsMYBc resulted in a reduction in NaCl-induced
164 expression of *OsHKT1;1* and salt sensitivity.

165

166 More progress has been made towards the understanding of SOS1 regulation. The
167 *SOS1* gene is up-regulated by salinity (Shi et al., 2003; Martinez-Atienza et al., 2007)
168 and, in *Arabidopsis thaliana*, the mRNA is stabilized by an ill-defined process mediated
169 by reactive oxygen species (ROS) (Chung et al., 2008). The transport activity of SOS1 is
170 enhanced by protein phosphorylation that counteracts autoinhibition (Quintero et al.,
171 2002; Quintero et al., 2011). The minimal SOS functional module in both dicots and
172 monocots comprises proteins SOS1, SOS2/CIPK24 and SOS3/CBL4 (Quintero et al.,

173 2002; Martinez-Atienza et al., 2007). SOS2/CIPK24 is a protein kinase that belongs to
174 the SnRK3 family (sucrose non-fermenting-1-related protein kinase-3). SOS3 is a
175 myristoylated Ca²⁺-binding protein that likely perceives the increase in cytosolic Ca²⁺
176 elicited by excess Na⁺ and recruits SOS2 to the plasma membrane to achieve
177 phosphorylation and activation of SOS1 (Quintero et al., 2002; Quintero et al., 2011).
178 In Arabidopsis, SOS3 functions primarily in roots whereas the related protein
179 SCaBP8/CBL10 operates by a similar mechanism in shoots (Quan et al., 2007). CBL10
180 may also regulate Na⁺ uptake into the vacuole (Kim et al., 2007).

181

182 The rice complement of SOS proteins has been characterized by heterologous
183 expression in yeast and Arabidopsis (Martinez-Atienza et al., 2007), but a precise
184 description of their role in the physiology and salinity stress management of rice plants
185 has not been achieved. Here we show that a *sos1* loss-of-function mutant displays
186 exceptional salt sensitivity that correlates with excessive Na⁺ intake and impaired Na⁺
187 loading into the xylem. We took advantage of the acute sensitivity of *sos1* plants to
188 Na⁺ to inspect the sodicity-stress transcriptome with little interference by the osmotic
189 challenge intrinsic to high salinity treatments. Surprisingly, *sos1* mutant roots showed
190 a marked down-regulation of genes despite the greater intensity of the stress they
191 suffered, which suggested impaired stress detection or an inability to mount a
192 comprehensive response to salinity stress in the absence of SOS1.

193

194

195 **RESULTS**

196

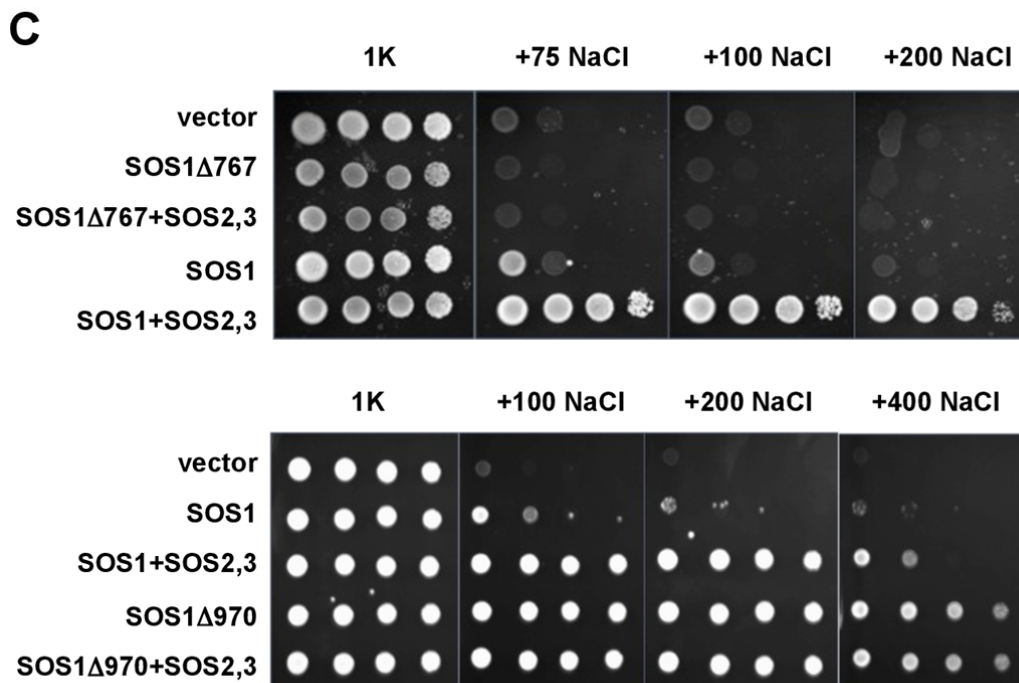
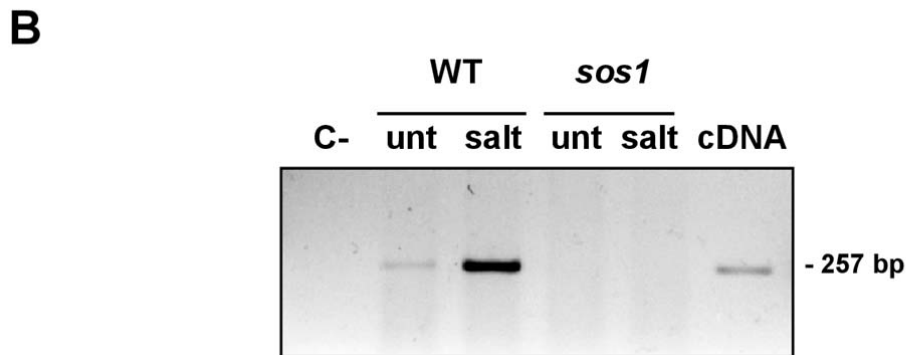
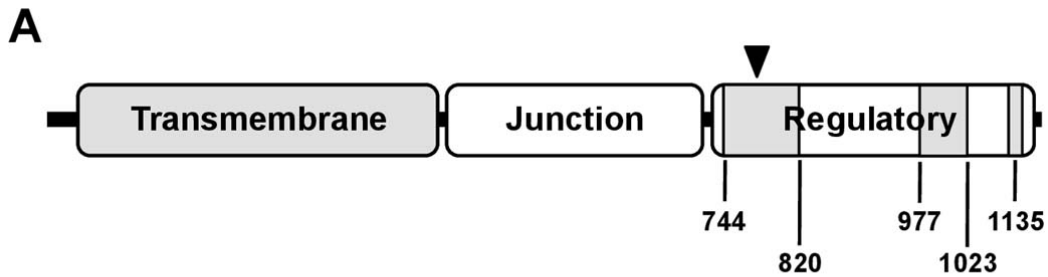
197 *Selection of mutant lines*

198

199 Public repositories of rice mutant lines were surveyed for entries with putative T-DNA
200 or Tos17 insertions in genes *SOS1* (Os12g44360), *CIPK24/SOS2* (Os06g40370) and
201 *CBL4/SOS3* (Os05g45810). Mutants in the Nipponbare genetic background were
202 preferred because this temperate *japonica* cultivar enabled the lines of interest to
203 complete their life cycle in our experimental paddies. Mutant line codes, annotated
204 insertion points of mutagenic DNA, and insertions experimentally confirmed by
205 diagnostic PCR and sequencing of amplicons are listed in Table 1. Detailed description
206 of the molecular characterization of all insertional lines tested herein is given in the
207 Supplemental Information file. As contrasting materials for mutant phenotyping,
208 congenic plants in which the mutagen was segregated out (null segregants) were kept
209 as controls for subsequent experimentation.

210

211 Out of seven mutant lines tested for putative mutagenic insertions at *SOS1* (Piffanelli
212 et al., 2007), only AVIB03 carrying an insertion of Tos17 at intron 19 was confirmed not
213 to produce a wild-type *SOS1* mRNA (Supplemental Figures S1 to S4). Molecular
214 analyses demonstrated that homozygous plants of this lineage produced a truncated
215 mRNA whose elongation was interrupted by the transposon and that, in contrast to
216 that in other lines (Supplemental Figures S1 to S4), salinity treatment did not promote
217 the correct processing of the mRNA and eviction of the Tos17 insertion (Figure 1B). Of
218 note is the significant induction of *SOS1* mRNA by salinity in Nipponbare plants.
219 Sequencing of the RT-PCR amplicon with the intervening Tos17 (Supplemental Figure
220 S4D) demonstrated that transposon insertion in AVIB03 had occurred after nucleotide
221 9802, relative to the ATG initiation codon in the genomic sequence of *SOS1*. The *in*
222 *silico* read-through translation of the putative mRNA predicted the synthesis of a *SOS1*
223 protein with a frame-shift after residue K767 and the addition of a short C-terminal
224 extension of sequence VCFCKTTLSH. This truncated protein was predicted inactive
225 based on the removal of essential amino acids and functional domains of the wild-type
226 protein that are downstream the truncation point (Figure 1A) (Quintero et al., 2011;



227 Nuñez-Ramirez et al., 2012). To demonstrate this point, a synonymous mutation was
 228 created in the rice *SOS1* cDNA by introducing a stop codon after residue K767.
 229 Expression of this truncated *SOS1* protein (*SOS1*-Δ767) in the yeast strain AXT3K that is
 230 devoid of all endogenous Na⁺ transporters and is salt-sensitive (Quintero et al., 2002)
 231 failed to restore salt tolerance upon the co-expression of *SOS2* and *SOS3* proteins of
 232 Arabidopsis (Figure 1C). We have shown elsewhere the competence of the Arabidopsis

233 SOS2/SOS3 complex to activate the rice SOS1 protein (Martinez-Atienza et al., 2007),
234 and Figure 1C illustrates this fact. By contrast, the rice SOS1 protein with a C-terminal
235 truncation after residue E970 yielded a hyperactive and SOS2/SOS3-independent
236 exchanger (Figure 1C). Truncation at residue E970 in the rice SOS1 protein mimics the
237 Arabidopsis mutant protein SOS1-Δ998, which lacks the autoinhibitory domain of SOS1
238 that is targeted by the SOS2/SOS3 kinase complex (Quintero et al., 2011).

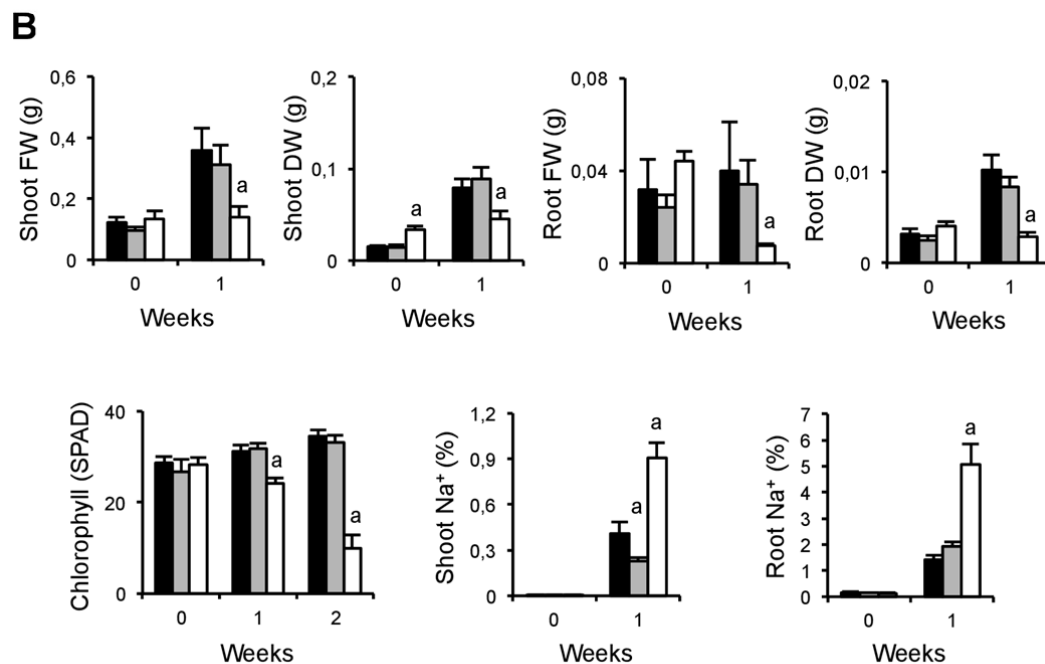
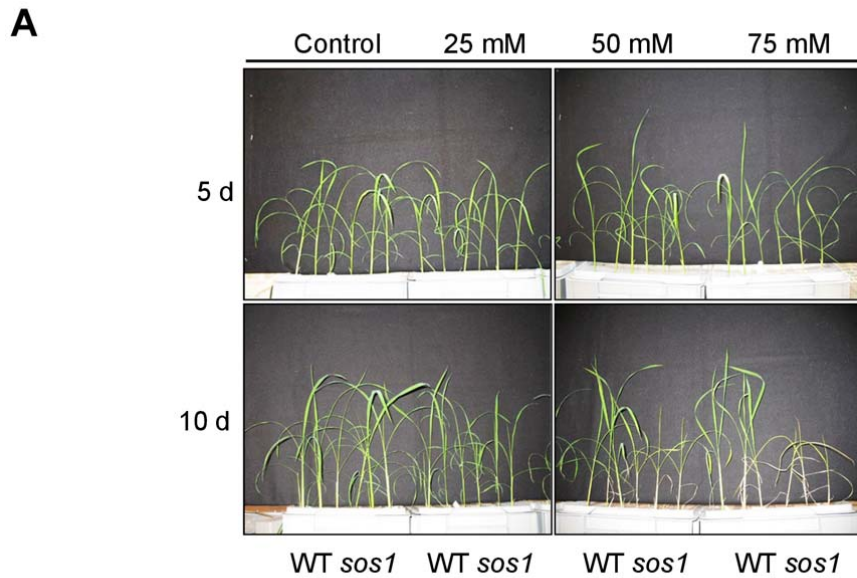
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240 In accordance with the yeast data demonstrating a defective SOS1 protein in line
241 AVIB03, plants of that line showed a remarkable salt-sensitive phenotype in
242 hydroponic culture that correlated with much greater Na⁺ contents in shoots and roots
243 (Figure 2). Representative pictures of *sos1* plants at different time points after stress
244 imposition (25, 50 and 75 mM NaCl) are shown in Figure 2A. The *sos1* mutant plants
245 showed growth retardation as early as 5 d after treatment with 50 and 75 mM NaCl.
246 After 10 d, *sos1* plants displayed severe symptoms of stress also at 25 mM NaCl, the
247 lowest salt concentration tested. The experiment was terminated after 24 d when all
248 *sos1* plants had died irrespective of the salt concentration. Quantitative growth data at
249 7 d after transfer to 50 mM NaCl confirmed the salt-sensitivity of homozygous *sos1*
250 plants compared to azygous and wild-type Nipponbare controls (Figure 2B). At that
251 point, *sos1* roots were severely dehydrated and after 14 d *sos1* shoots had bleached.
252 Salt sensitivity correlated with greater Na⁺ contents in roots and shoots of the *sos1*
253 mutant.

254

255 The SOS1 protein activity accounts for ca. 60% of the Na⁺/H⁺ exchange measured in
256 plasma membrane vesicles of Arabidopsis (Qiu et al., 2003). Measurement of Na⁺/H⁺
257 exchange in purified plasma membrane fractions (Supplemental Figure S5)
258 demonstrated 39% reduction in exchange activity in vesicles derived from the AVIB03
259 line relative to that in the wild-type plants. Quenching of fluorescence units per mg of
260 protein per minute was 34.73 ± 2.56 and 56.74 ± 11.34, respectively ($p=0.107$ by One-
261 Way ANOVA test, $n=4$ technical replicas in two different assays). Together, these
262 results signify that line AVIB03 is deficient in SOS1 activity and hence this lineage was
263 selected for further experimentation.

264



265 We next searched for mutants in the rice genes *CIPK24* and *CBL4*, which we have
 266 previously described as functional homologues of Arabidopsis *SOS2(CIPK24)* and
 267 *SOS3(CBL4)* (Martinez-Atienza et al., 2007). Mutant lines in the Nipponbare
 268 background AMHC11, AMHC12 and AMHE12 were annotated to carry T-DNA
 269 insertions 84 nucleotides upstream of the translation initiation codon of locus
 270 Os06g40370 (*CIPK24*) (Sallaud et al., 2004). Molecular characterization confirmed
 271 identical T-DNA insertion points at the 5'-UTR of *CIPK24* in these lines (Supplemental

272 Figure S6). Homozygous plants produced a mature *CIPK24* mRNA carrying the T-DNA
273 insertion and no wild-type mRNA was detected. Mutant seedlings were sensitive to
274 NaCl in hydroponic culture. Supplemental Figure S6 illustrates the end-point growth of
275 homozygous *cipk24* plants (L24H), azygous *CIPK24* controls (L24A), and wild-type
276 Nipponbare plants after 7 d in hydroponic medium supplemented with 40 and 80 mM
277 NaCl. These data indicate that the rice kinase CIPK24, which has been shown to
278 activate SOS1 (Martinez-Atienza et al., 2007), also plays a role in the salt tolerance of
279 rice.

280

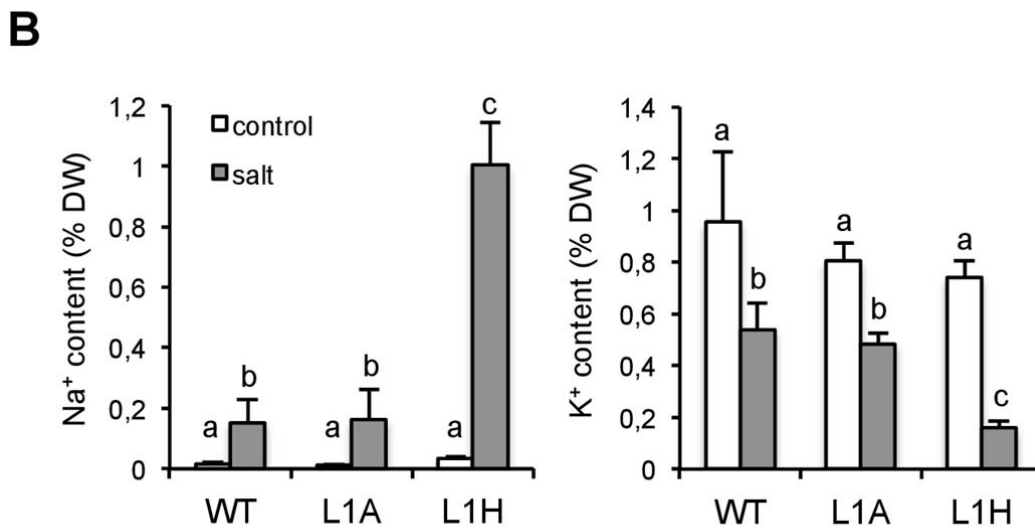
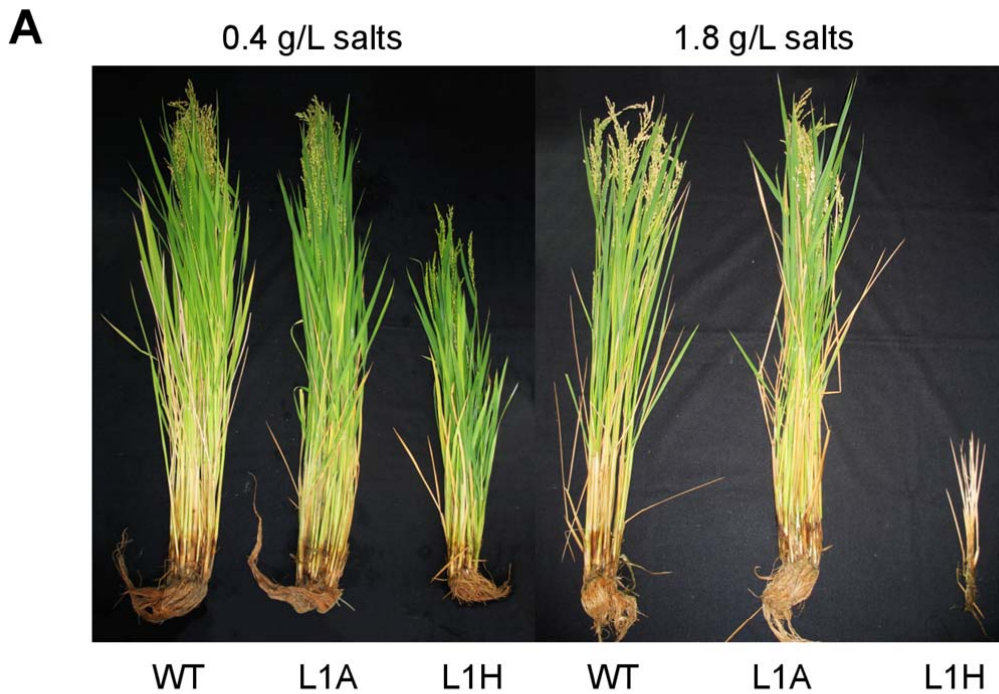
281 At the time this research was conducted, there were no available mutants with
282 mutagenic insertions in the coding region of gene *CBL4* (Os05g45810) in the
283 Nipponbare background. Line AJBC12 was annotated to bear a T-DNA insertion right
284 downstream the 3'-UTR of *CBL4* (Sallaud et al., 2004). RT-PCR analyses with T2 plants
285 of this lineage showed that the *CBL4* transcript was not affected by the adjacent T-DNA
286 insertion and no further experimentation was done with this line.

287

288 *Salt tolerance assays in rice paddies*

289

290 To examine in detail the relevance of the SOS system in the salt tolerance of cultivated
291 rice, we next sought to define the performance of *sos1* and *cipk24* mutant plants in
292 near-agronomical conditions in experimental rice paddies. Homozygous mutant lines
293 of genotype *sos1* and *cipk24* (denoted henceforth as L1H and L24H, respectively) were
294 compared to congeneric segregant lines selected during the genotyping as lacking the
295 mutagenic insertion (denoted here as azygous segregant lines L1A and L24A), and to
296 control plants of the Nipponbare background. In lines L1H and L1A the T-DNA of the
297 original transformation was also segregated out. Four-week-old plants were
298 transferred to paddies irrigated with water from an underground well whose salinity
299 content was on average 0.42 g/L, of which Na⁺ was at 5.09 mM, K⁺ at 0.14 mM, and
300 Ca²⁺ at 4.0 mM. For the saline treatment, underground water was supplemented with
301 seawater salts to a final concentration of 1.8 g/L salts during irrigation to mimic
302 frequent salinity episodes in rice farms in Spain. The final concentrations of Na⁺, K⁺ and
303 Ca²⁺ in the salinized irrigation water were 27.6, 0.24 and 4.1 mM, respectively.



304 Chloride was 30.5 mM. As depicted in Figure 3 and Supplemental Figure S7, *sos1* plants
 305 showed statistically significant growth retardation even at the low salt content of
 306 irrigation water, conditions in which the Na⁺ content in *sos1* shoots was low but still
 307 triple that of the congenic *SOS1* control and the wild type Nipponbare (0.035% vs
 308 0.01% of dry matter, respectively). Growth arrest of salinized *sos1* plants at 1.8 g/L salt
 309 correlated with massive Na⁺ accumulation in shoots and notable K⁺ loss (Figure 3). The
 310 *cipk24* mutant plants showed a modest growth reduction in these conditions that was
 311 statistically significant only at $p = 0.095$ ($n = 10$) relative to that in the congenic azygous

312 control (Supplemental Figure S7), in agreement with the salt sensitivity that mutant
313 *cipk24* showed at early developmental stages in hydroponic culture at 80 mM NaCl but
314 not at 40 mM (Supplemental Figure S6).

315

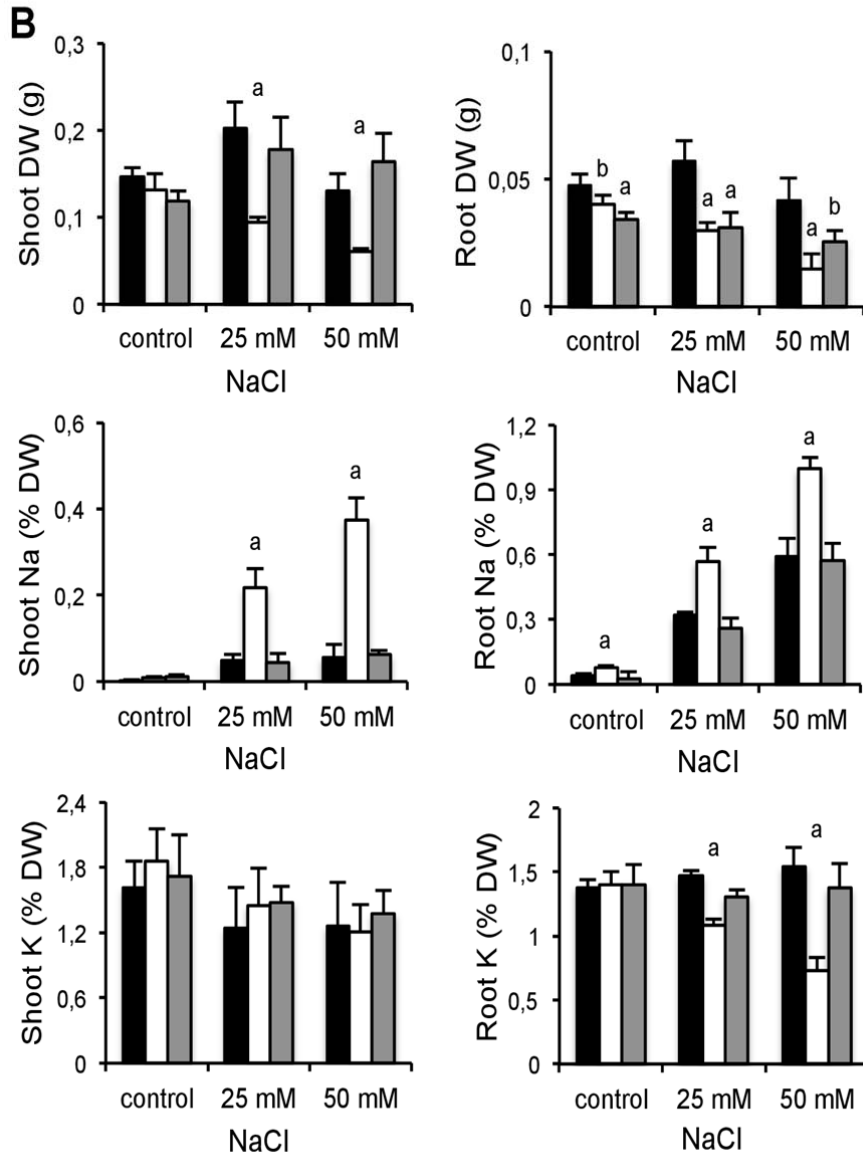
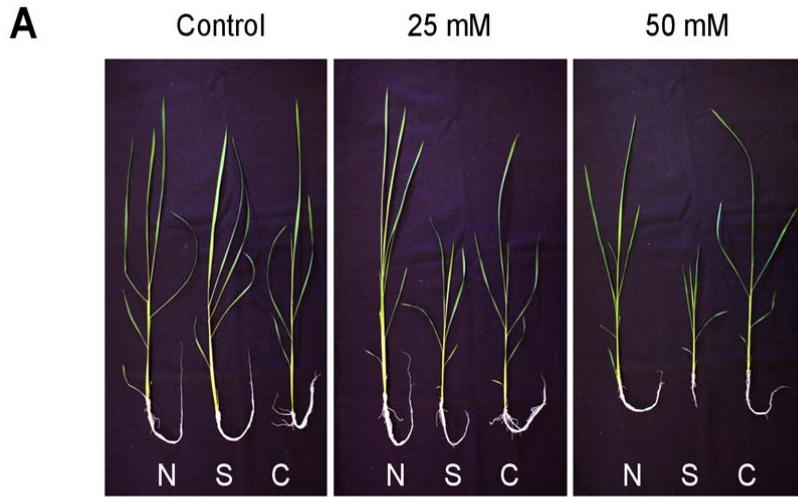
316 *Complementation and over-expression lines*

317

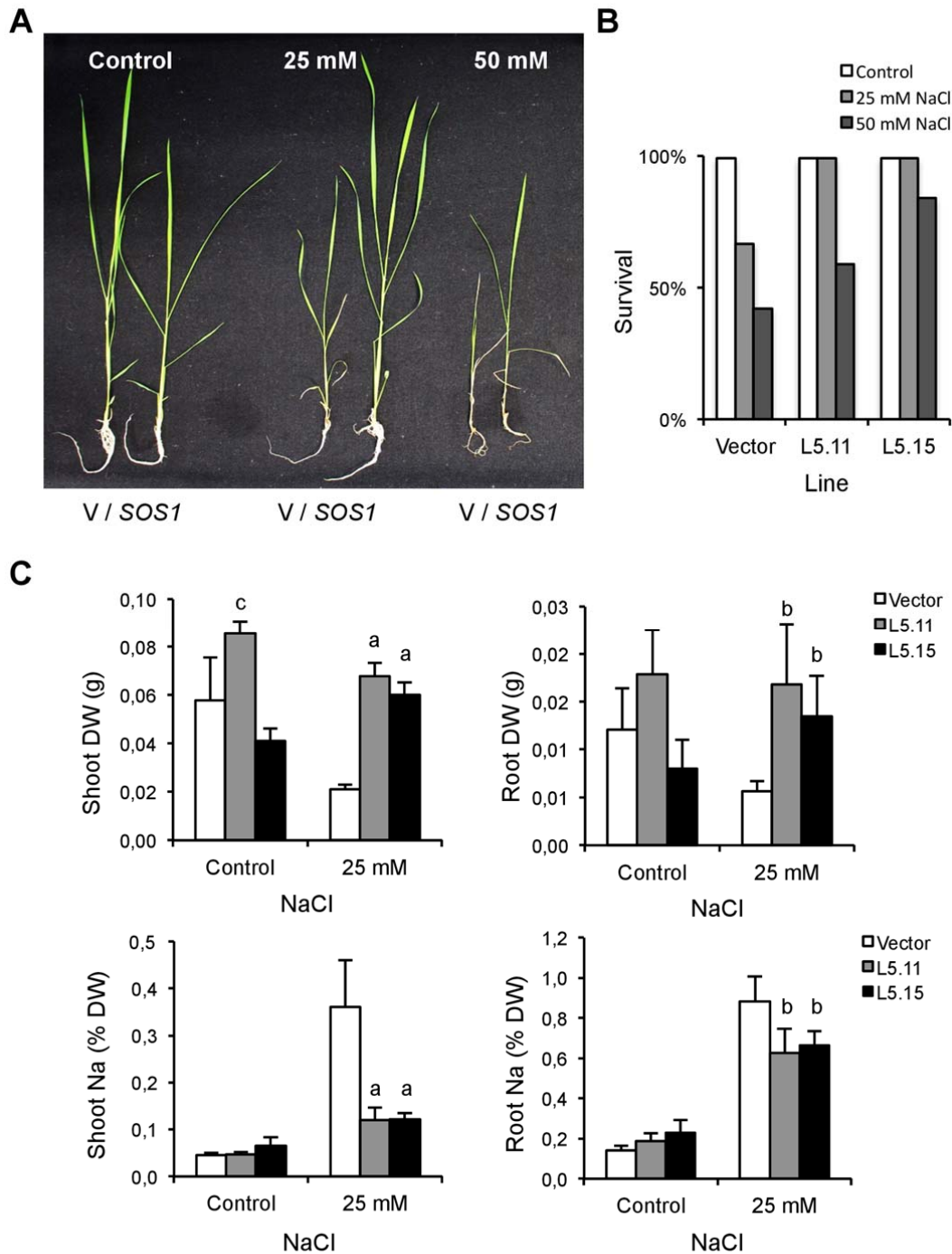
318 Because only one putative *sos1* mutant line was available, we sought to confirm that
319 the exceptional salt-sensitivity of AVIB03 plants was due to the lack of function of *SOS1*
320 and not to another uncharacterized mutation carried over through the mutant
321 selection process. Genetically complemented lines were obtained by transformation of
322 AVIB03 plants with the *SOS1* cDNA under the control of the constitutive and
323 moderately expressed *UBI1* gene promoter of maize. Restoration of salt-tolerance was
324 confirmed by scoring the growth of geneticin-resistant T1 plants (n=32), belonging to
325 four independent transformation lines, in hydroponic culture with 50 and 75 mM NaCl
326 for 3 weeks (Supplemental Figure S8A). This test showed that 13 out of 16 plants re-
327 gained tolerance to 50 mM NaCl, and that 12 out of 16 withstood the 75 mM NaCl
328 treatment. None of the untransformed *sos1* plants survived the assay (n=8). To further
329 confirm the basis for the suppression of the salt-sensitive phenotype, the
330 complemented line C11 that had a low expression level of the *UBQ1:SOS1* transgene
331 (Supplemental Figure S8B) was chosen for additional analyses. Two-week-old seedlings
332 of wild type, *sos1* mutant and transformed line C11 were transferred to 25 and 50 mM
333 NaCl in Miyamoto's medium for 3 weeks, after which plant weight, and Na⁺ and K⁺
334 contents were determined. Results showed that shoot growth in saline media was
335 restored to wild-type levels, whereas root growth lagged behind that of control plants
336 (Figure 4). Moreover, Na⁺ content in C11 plants largely matched that in wild-type
337 plants.

338

339 To test the potential of the *SOS1* exchanger to increase the salt tolerance of rice, we
340 also generated Nipponbare transgenic plants with expression of the *SOS1* cDNA driven
341 by the strong CaMV 35S gene promoter to create *SOS1* overexpression. Transgenic
342 plants showing high levels of transgene expression as determined by RT-PCR were
343 selected and propagated to produce homozygous lines. Salt tolerance was evaluated in



344 hydroponic cultures with Miyamoto's medium, which contains low levels of K^+ (0.20



345 mM) and Na^+ (0.14 mM) and thus could maximize the impact of the ionic component
 346 of moderate salt stress (25–50 mM NaCl). Seven-d-old seedlings of two independent
 347 35S:*SOS1* lines were cultivated in the presence of 25 and 50 mM NaCl for 4 weeks (n=5
 348 per genotype and treatment). Transgenic plants overexpressing *SOS1* showed greater
 349 survival under salt treatments than that of control plants transformed with a mock

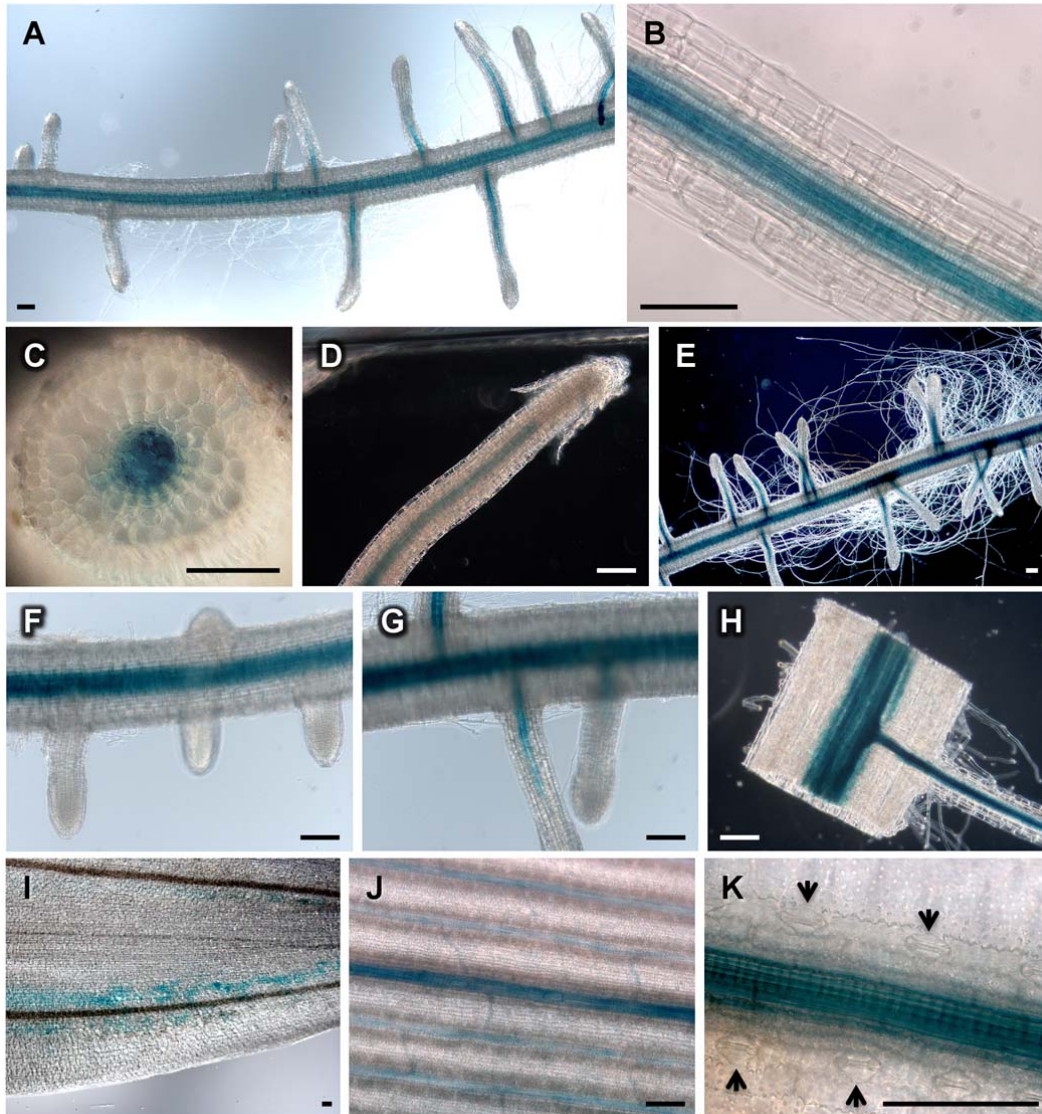
350 empty vector (Figure 5). Plants that survived the treatment at 25 mM were assessed
351 for dry weight and Na⁺ content (control plants surviving 50 mM NaCl had damaged
352 roots and were discarded for further analyses; see Figure 5A). Plants overexpressing
353 *SOS1* sustained a robust growth at 25 mM NaCl that correlated with lower Na⁺
354 contents in both shoots and roots compared to that in control plants (Figure 5C), as
355 should be expected from the near ubiquitous expression of *SOS1* driven by the 355
356 promoter and the consequently enhanced Na⁺ efflux in many cell types.

357

358 *Expression pattern of SOS1*

359

360 In dicots, the *SOS1* protein has been implicated in Na⁺ fluxes relevant to net ion uptake
361 by roots and to xylem loading for root/shoot ion partition (Shi et al., 2002; Oh et al.,
362 2009; Olias et al., 2009). To understand the critical role of *SOS1* in the salt tolerance of
363 rice plants, the expression pattern of the *SOS1* gene was investigated in transgenic rice
364 transformed with the *SOS1* gene promoter (defined as the 3.9 kb genomic fragment
365 upstream the initiation codon) fused to the β-glucuronidase (*GUS*) reporter gene. Four
366 independent transgenic lines were assayed for *GUS* expression and they all produced
367 consistent staining patterns, thereby ruling out artifacts by positional effects of the
368 transgene. *GUS* expression was detected at all developmental stages tested and no
369 significant differences in the expression pattern were found between control and
370 saline conditions (50 mM NaCl, 24 h). Maximal *GUS* staining occurred in the
371 vasculature, both in shoots and roots (Figure 6). In roots, longitudinal and transverse
372 sections indicated a preferential expression in xylem parenchyma (Figure 6A-C). No
373 expression was detected at the root apex or root hairs (Figure 6D,E). In secondary
374 roots the staining was apparent only after the vascular bundle started to differentiate
375 (Figure 6F-H). Likewise, the expression of *SOS1:GUS* was located in vascular bundles in
376 aerial tissues. In the coleoptile, staining was restricted to undifferentiated cells around
377 the two vascular bundles (Figure 6I). In the flag leaf, all vascular bundles were stained,
378 although the signal was stronger around older xylem vessels (Figure 6J,K). There was
379 no detectable expression in stomata (Figure 6K). Thus, the preferential expression
380 pattern of rice *SOS1* recapitulated the pattern described in Arabidopsis, with the
381 exception that the rice reporter gene *SOS1:GUS* was not strongly expressed in root tips



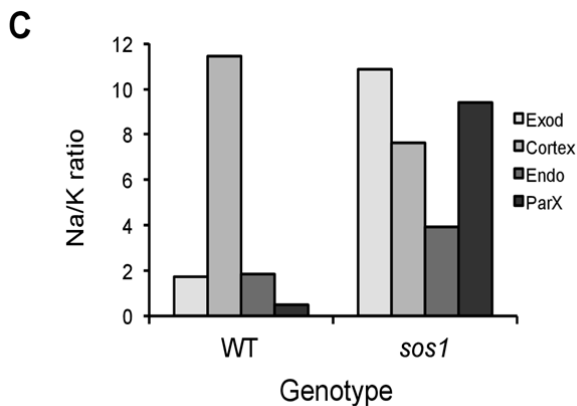
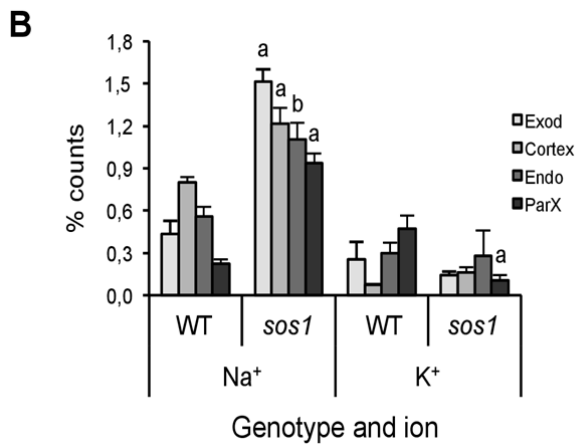
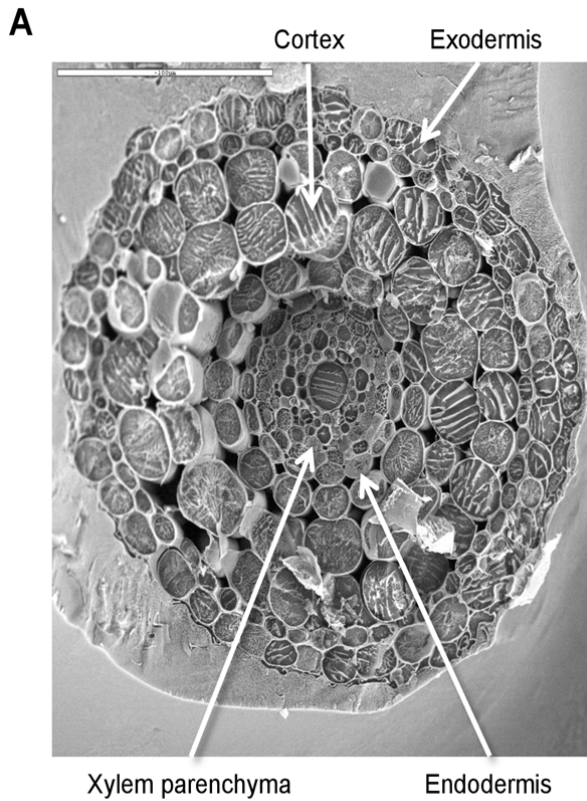
382 as reported for *Arabidopsis* plants using an equivalent approach (Shi et al., 2002). In
 383 conclusion, the expression pattern in rice suggested a role for *SOS1* in Na^+ xylem
 384 loading in addition to the cellular-based protection imparted by Na^+ efflux.

385

386 *Function of SOS1 in Na^+ efflux and xylem loading*

387

388 The preferential expression pattern of the *SOS1* gene promoter in parenchyma cells
 389 associated to the vasculature suggested a function for *SOS1* in xylem loading since, at
 390 physiological Na^+ and H^+ concentrations, a plasma membrane Na^+/H^+ exchanger likely
 391 mediates the energetically uphill Na^+ efflux out of parenchyma cells. Scanning Electron



392 Microscopy coupled to Energy Dispersive X-ray microanalysis (SEM-EDX) was used to

393 determine the mineral composition of various root cell types in cross-sections of salt-
394 treated rice plants (Figure 7). In parallel, net tissue contents of Na^+ and K^+ were also
395 determined. Roots of the *sos1* plants treated with 50 mM for 2 d in hydroponic culture
396 had a Na^+ content that doubled the amount in the wild type (112.95 mM vs. 51.4 mM),
397 and showed a 37% reduction in the K^+ content (10.0 mM vs 15.8 mM). In agreement
398 with these values, the amount of Na^+ measured by SEM-EDX in the *sos1* mutant was
399 greater than that in the corresponding wild-type samples of all cell types tested (Figure
400 7). In wild-type roots, the maximal Na^+ content was found in the cortex, with a
401 progressive decline in endodermis and xylem parenchyma cells. This profile is in
402 accordance with the selectivity filter imposed by the root endodermis (and buildup of
403 Na^+ content in the cortex relative to that in the endodermis), and with the active
404 loading of Na^+ ions into the xylem vessels after ions entered the stele (minimal Na^+
405 content in xylem parenchyma). By contrast, the Na^+ distribution in the *sos1* root
406 followed an inward-decreasing gradient signifying the unrestricted transit of Na^+
407 through all cell layers and the passive leak of Na^+ into the xylem. Together, these
408 results highlight the critical role that the Na^+/H^+ exchanger SOS1 plays in Na^+ efflux and
409 xylem loading in rice plants.

410

411 On the other hand, the minimal K^+ content in cortical cells in wild-type roots and the
412 increasing K^+ values towards the xylem reflected the active uptake of K^+ by
413 endodermal cells and its translocation towards the vasculature. A key parameter of the
414 physiological status of individual cells and whole plants under salt stress is the Na^+/K^+
415 ratio. Figure 7B shows that the ion-selective barrier imposed by the endodermis to the
416 centripetal movement of Na^+ in the root caused a disproportionate Na^+/K^+ ratio of
417 11.43 in cortex cells of wild-type roots, in stark contrast with the low 0.49 ratio
418 measured in xylem parenchyma cells presumably effected by the active Na^+ loading in
419 xylem vessels. In accordance with the critical role of SOS1 in Na^+ export to the xylem,
420 the Na^+/K^+ ratio in the xylem parenchyma of the *sos1* mutant escalated to 9.40, a 19-
421 fold increase relative to wild-type values. This change in the Na^+/K^+ ratio between wild
422 type and the *sos1* mutant signified the greatest difference among cell types. These
423 results are also evidence of the profound effects that a deficient Na^+ -specific transport
424 system may produce on the K^+ status of individual cells and tissues.

425

426 *Transcriptome of the sos1 mutant*

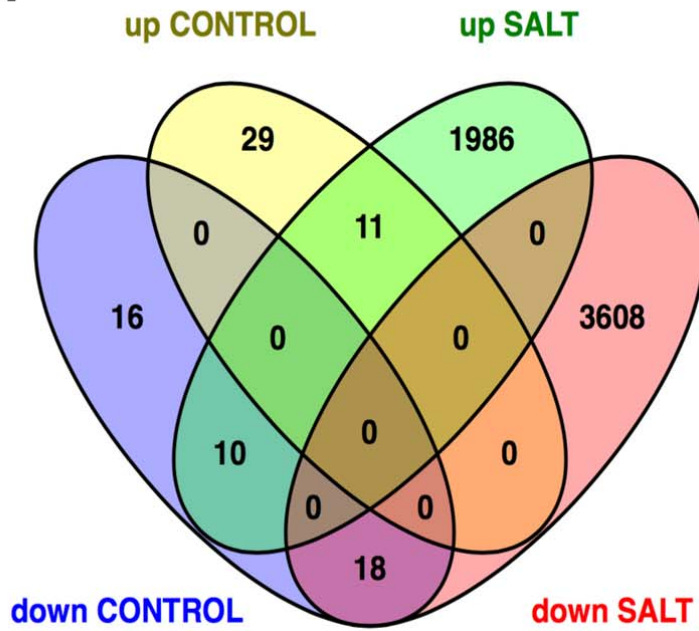
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428 High salinity imposes both a water stress because the osmotic effect of the saline
429 solution outside and an ionic imbalance resulting primarily from the accumulation of
430 Na^+ and the loss of K^+ (Munns and Tester, 2008). Contrary to most analyses of the
431 transcriptional response of plants to high-salinity, in which it is not possible to
432 separate the genetic response to the osmotic and the ionic components of high salinity
433 (Rabbani et al., 2003; Kanwar et al., 2014; Wang et al., 2016), the extreme Na^+ -
434 sensitivity of the *sos1* mutant plant offered the unique advantage of inspecting the
435 specific transcriptional response to sodicity stress at low external salt concentrations.
436 Roots are the main defensive barrier against salinity and the organ that first senses this
437 soil-derived stress (Munns and Tester, 2008). Hence, to identify genes responding
438 primarily to the Na^+ toxicity affecting *sos1* plants, roots from mutant and control wild-
439 type seedlings were harvested at the same time point (4 d) after a 75 mM NaCl
440 treatment, and compared to roots harvested alongside from untreated seedlings. Total
441 RNA was extracted and used for microarray experiments (see “Materials and
442 Methods” for details).

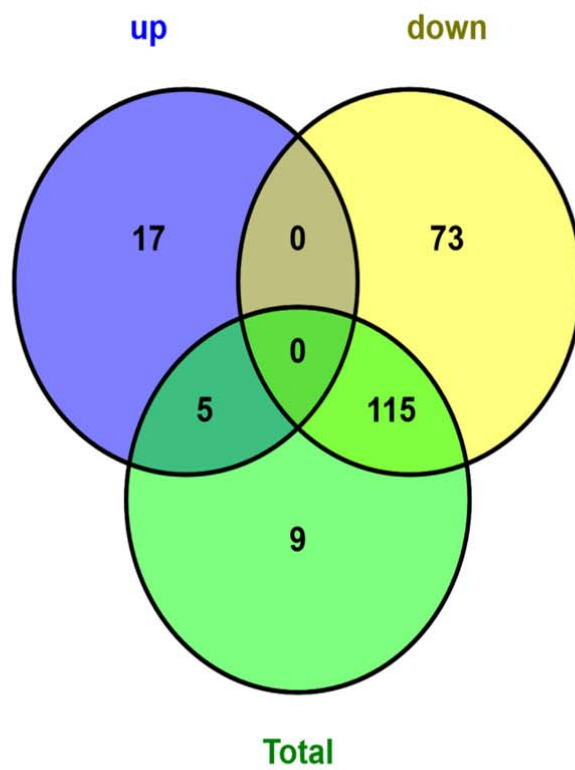
443

444 Based on the standard criteria of greater than 2-fold changes with statistical
445 significance at $p < 0.05$ in *t*-tests, we identified differentially regulated genes (DEGs) in
446 *sos1* mutant roots under salt and control conditions. Supplemental Table S1 online
447 summarizes the genes up- and down-regulated in the mutant roots when compared to
448 expression levels in the control line under the two treatments. Only 84 DEGs,
449 representing 0.3% of total genes annotated, were detected between the two
450 contrasting lines cultivated in control conditions. This result points to a minor role of
451 SOS1 during the root development in the absence of sodicity stress. Of note is that
452 *HKT2;1* (Os06g0701700), encoding a transporter protein that mediates Na^+ influx into
453 K^+ -starved roots and thereby improves growth (Horie et al., 2007), was the second
454 most up-regulated DEG in the *sos1* mutant without salinity treatment (Supplemental
455 Table S1). By contrast, many more genes were differentially expressed in the mutant
456 relative to that in the wild type when salt stress was imposed, in accordance with the

A



B



457 substantial impact of salinity on *sos1* roots. Of the 5678 total DEGs identified in the

458 roots of the *sos1* mutant under salinity, only 11 genes were commonly up-regulated
459 and 18 were down-regulated in the *sos1* mutant relative to expression levels in the
460 wild type irrespective of the salt treatment (Figure 8), indicating that the
461 transcriptional impact of the *sos1* mutation was largely specific to the salt-induced
462 response. The large number of DEGs (5633 genes, 19.48% of total) that presented an
463 altered response in *sos1* roots relative to that in wild-type roots under salt stress
464 demonstrates the drastic effect of the *sos1* mutation in the transcriptional response to
465 sodicity. Notably, the majority (64%, 3608 genes) of these salt-responsive DEGs were
466 down-regulated in the mutant root, which may represent a biased transcriptional
467 response towards either repressed or less-induced transcription in the *sos1* mutant in
468 response to salt.

469

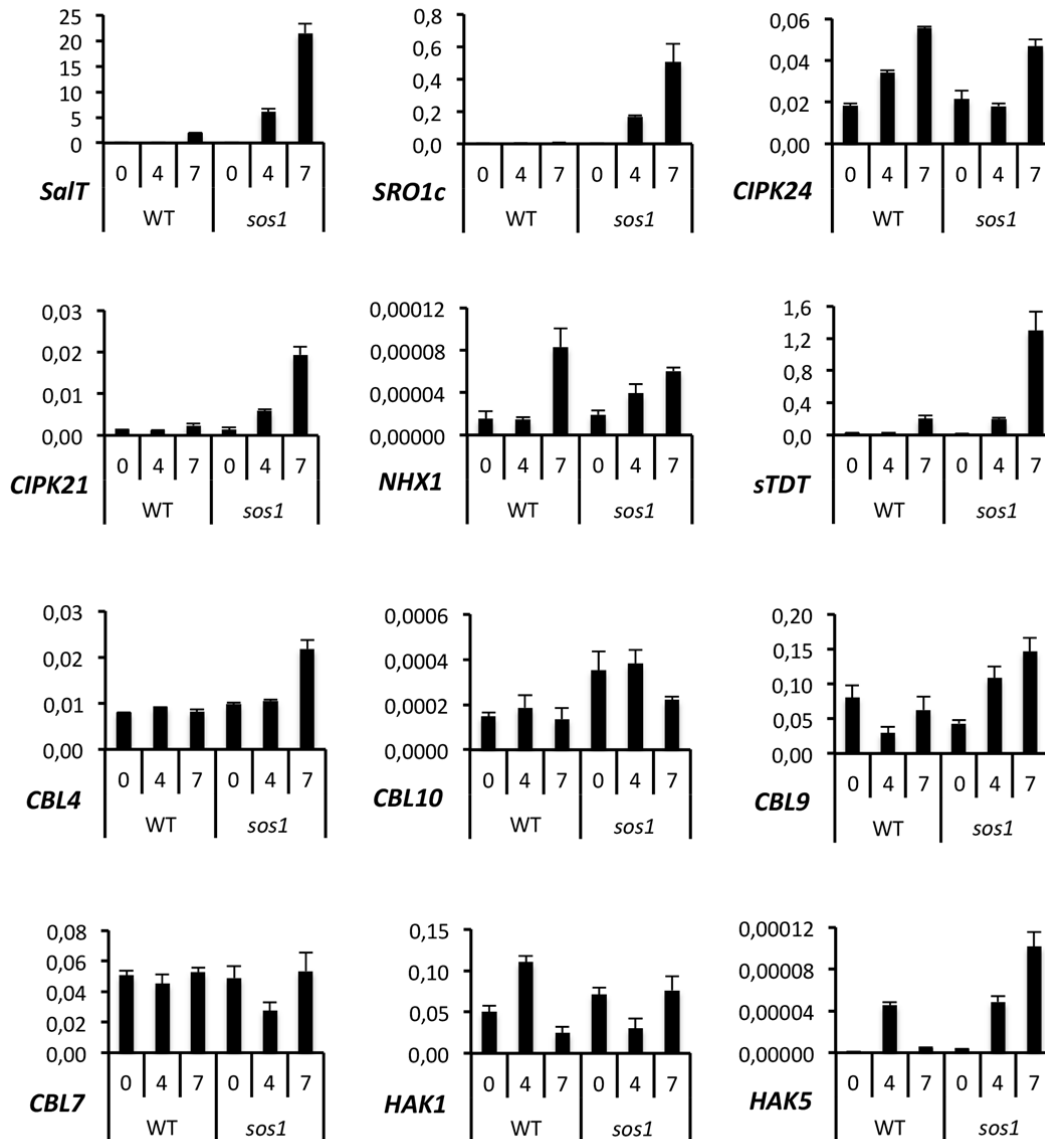
470 To evaluate the potential biological function of the differentially expressed genes in
471 the *sos1* mutant, Gene Ontology (GO) analysis was performed using the AgriGO online
472 service (<http://bioinfo.cau.edu.cn/agriGO>) for the up-regulated, down-regulated and
473 total DEGs identified in the salt-stressed or control-cultivated roots (Supplemental
474 Table S2). The 5633 salt-responding DEGs fell into 129 significantly enriched GO
475 categories corresponding to 66 biological processes, 28 functional categories, and 35
476 cellular components. The top over-represented GO categories included "cellular
477 nitrogen compound metabolic process" (137 genes; False Discovery Rate (FDR) of
478 7.2×10^{-16}), and those related to the biological process "transport" (FDR = 2.2×10^{-4})
479 (Supplemental Table S2). Further analysis showed again that these GO categories were
480 constituted by far more down-regulated than up-regulated genes. This bias explained
481 that most (n=115) of the 129 significant GO-terms were also enriched specifically in
482 the group of the down-regulated DEGs (Figure 8), including the 25 top enriched GO
483 categories (Supplemental Table S3). Moreover, 73 of the significant GO terms
484 identified were only present among the enriched categories of the down-regulated
485 DEGs. For example, among the 97 total DEGs classified under the term "ion
486 transmembrane transporter activity" (GO:0015075), 79 of them were down-regulated
487 DEGs. The GO cellular component "membrane" and "membrane part" ranked top
488 among GO terms enriched in down-regulated DEGs.

489

490 The MapMan software (Thimm et al., 2004) was also used to assign genes to functional
491 categories and to further analyze the metabolic pathways and cellular responses
492 altered in *sos1* roots. Over-representation analysis of the up- and down-regulated
493 categories (Supplemental Table S4 and Supplemental Figure S9) indicated a general
494 repression of the glycolysis, the tricarboxylic acid (TCA) cycle, nitrogen metabolism,
495 amino acid synthesis, redox metabolism, protein targeting (secretory pathway) and
496 protein degradation (ubiquitin proteasome). Among the genes related to “abiotic
497 stress” responses, only the “drought/salt” and “unspecified” sub-categories were
498 significantly different between the two genotypes, with an over-representation of
499 unspecified stress genes among the up-regulated salt-responding DEGs. Together,
500 these results support the notion that the transcriptional response of the *sos1* mutant
501 to salt involves a global alteration of the cell physiology due to preferential down-
502 regulation of genes that otherwise would be part of the normal response of wild-type
503 roots to salinity.

504

505 The majority of the DEGs showing the largest variations in transcript abundance
506 corresponded to proteins of unknown function or with sequence similarity to other
507 proteins but whose specific function remains to be determined. Moreover, the
508 differential hybridization method used in microarrays only captures genes whose
509 transcript abundance at sampling are substantially different between samples; genes
510 whose transcriptional dynamics are of similar magnitude between samples are not
511 detected even if they responded to the stimulus. This was best exemplified by *CIPK24*,
512 which presumably was not identified as a DEG by the microarray data because this
513 gene was similarly induced in wild-type and *sos1* roots, as demonstrated by RT-qPCR
514 (Figure 9). Thus, we analyzed in greater detail groups of genes encoding proteins with
515 a known role in processes that are likely to be relevant to the ionic component of
516 salinity stress, mainly Na⁺ and K⁺ transporters and their regulators CIPKs and CBLs.
517 Among the 100 genes selected for this analysis (Supplemental Table S5), the rice *SalT*
518 locus was the most transcriptionally induced gene in *sos1* roots relative to that in
519 control salt-treated roots (log₂ FC = 4.79). Gene *SalT*, encoding a 15-kDa jacalin-related
520 mannose-binding lectin, is located on a QTL responsible for the bulk of genetic
521 variation in ion uptake under saline conditions at the seedling stage (Thomson et al.,



522 2010). The strong induction of this gene was confirmed by RT-qPCR (Figure 9).
 523 Likewise, *SRO1c* (Os03g0230300) was highly induced in *sos1* roots in the
 524 transcriptomic data set (\log_2 FC = 4.56) and verified by RT-qPCR (168-fold induction at
 525 7 d, compared to 2.6-fold in the wild type). *SRO1c* encodes a putative poly-[ADP-
 526 ribose] polymerase (PARP) and is the most stress-responsive gene within the rice *SRO*
 527 family (You et al., 2013). Notably, *SRO1c* is closely related to the Arabidopsis protein
 528 RCD1, an inactive PARP that shuttles between nucleus and cytoplasm to interact with
 529 SOS1 (Katiyar-Agarwal et al., 2006).
 530

531 Among the CIPK-encoding genes, *CIPK21* showed the largest transcriptional up-
532 regulation by salt in *sos1* vs. wild-type roots according to the microarray data (\log_2 FC =
533 2.22), which was confirmed by RT-qPCR. A modest 1.93-fold transcriptional induction
534 was measured in wild-type roots after 7 d in salt, whereas in *sos1* the 6.05-fold
535 induction after 4 d treatment was followed by a striking 21.47-fold induction after 7 d
536 (Figure 9). *CIPK15*, which has been implicated in the salt stress response and enhances
537 the salinity tolerance of transgenic rice plants (Xiang et al., 2007), was also up-
538 regulated in the *sos1* mutant (\log_2 FC = 1.75; Supplemental Table S5). *CIPK1*, *31*, *10* and
539 *17* also showed differential up-regulation in *sos1* roots relative to that in the wild type,
540 whereas *CIPK23*, *29*, *25* and *4* were down-regulated by at least 2-fold. *CBL* genes
541 showed little differential responsiveness in microarray data, with *CBL9* being the most
542 highly induced (\log_2 FC = 2.04) and *CBL7* the most down-regulated (\log_2 FC = -2.46). RT-
543 qPCR data confirmed the up-regulation of *CBL9* and showed that the down-regulation
544 of *CBL7* in *sos1* was only transient at 4 d, with a return to pre-stress level after 7 d
545 (Figure 9). Although not identified as statistically significant DEGs, the rice homologs of
546 *CBL4/SOS3* and *CBL10*, the two CBLs regulating *CIPK24/SOS2* and *SOS1* in Arabidopsis,
547 showed complementary kinetics in *sos1* roots so that the transient induction of
548 *OsCBL10* was followed by up-regulation of *OsCBL4* (Figure 9).

549

550 Successful adaptation to salinity requires the accumulation of inorganic and organic
551 osmolytes inside vacuoles for osmoregulation and ion detoxification (Munns and
552 Tester, 2008). Among these osmolytes, Na^+ , K^+ , Cl^- and organic acids such as malate are
553 most abundant. In Arabidopsis, *NHX1* and *NHX2* are vacuolar Na,K/H antiporters that
554 function *in planta* to drive the uphill accumulation of K^+ into the vacuolar lumen for
555 osmotic regulation, generation of cellular turgor, and stomatal movements (Leidi et al.,
556 2010; Barragan et al., 2012; Andrés et al., 2014). The rice genes encoding exchangers
557 *OsNHX1* and *OsNHX2* showed a 3- to 4-fold transcriptional up-regulation in *sos1* roots.
558 Gene Os09g0484900, encoding a putative protein 75% similar to the tonoplast
559 dicarboxylate transporter of Arabidopsis involved in the uptake of malate into the
560 vacuole (Emmerlich et al., 2003), was also highly induced in the salinized *sos1* roots
561 according to both the microarray and RT-qPCR data (8-fold induction at 7 d in the WT
562 vs. 85-fold in *sos1*). Malate storage within the vacuole allows the plant to accumulate

563 this metabolite to very high concentrations (up to >300 mM) and this large gene
564 induction likely reflects the need to accumulate anions in the vacuolar lumen for
565 osmotic adjustment and charge compensation of compartmentalized Na⁺. These
566 results suggest that in response to salinity, the *sos1* mutant differentially modulates
567 multiple K⁺ and malate transporters to achieve cytosolic ion homeostasis and
568 osmoregulation in intracellular compartments.

569

570 Other genes encoding K⁺-transport proteins showed minor transcriptional responses.
571 No *HAK* genes showed differential up-regulation in *sos1* roots compared to that in the
572 wild type according to microarray data. On the other hand, *HAK1*, 5 and 16 were
573 down-regulated 3- to 4-fold in *sos1* relative to that in the wild type. *OshAK5* mediates
574 high-affinity K⁺ uptake by roots under nutrient limitation and in saline conditions (Yang
575 et al., 2014), whereas *OshAK1* plays a role in K⁺ uptake and translocation to shoots
576 over low- and high-K⁺ concentration ranges (Banuelos et al., 2002; Chen et al., 2015).
577 Closer inspection by RT-qPCR showed that both *OshAK1* and *OshAK5* transcript
578 abundance displayed complex kinetics (Figure 9). *OshAK1* transcripts showed opposite
579 kinetics after stress imposition in control and *sos1* plants. A transient induction of
580 *OshAK5* was also observed in control plants, but the up-regulation of *OshAK5* was
581 sustained throughout the treatment in the *sos1* mutant. The RT-qPCR data is in
582 agreement with the expected role of *OshAK5* in sustaining K⁺ uptake in a saline
583 environment (Horie et al., 2011; Yang et al., 2014) and probably reflects the need of
584 *sos1* roots to re-establish K⁺ homeostasis under salinity.

585

586 The HKT proteins of cereals have been involved in the uptake of Na⁺ by roots and in
587 the root-to-shoot partition of Na⁺ and K⁺ (Garcia-deblas et al., 2003; Yao et al., 2010;
588 Munns et al., 2012; Hamamoto et al., 2015). Out of eight *HKT* genes in the rice
589 genome, only *HKT8/HKT1;5/SKC1* (Os01t0307500) showed a modest yet statistically
590 significant down-regulation in *sos1* roots under salt (log₂ FC = -0.81), whereas
591 *HKT1/HKT2;1* (Os06t0701700) was up-regulated in *sos1* roots in the absence of stress
592 (log₂ FC = 2.10). The rice transporter HKT8/SKC1 regulates Na⁺/K⁺ levels in shoots by
593 recirculating Na⁺ from shoots to roots (Ren et al., 2005). The significance of the
594 *HKT8/HKT1;5/SKC1* down-regulation might be related to the excessive accumulation of

595 Na^+ in the *sos1* roots and represent a root-protecting mechanism limiting the back-
596 flow of Na^+ to roots.
597
598

599 **DISCUSSION**

600

601 We showed previously that the rice SOS system comprises the OsSOS1 transporter, the
602 OsCIPK24 protein kinase that is the closest homologue to the Arabidopsis
603 SOS2/AtCIPK24, and OsCBL4 that is most similar to SOS3/AtCBL4 (Martinez-Atienza et
604 al., 2007). The fact that rice OsSOS1 is encoded by the single copy gene Os12g44360
605 allowed a reverse-genetics approach to determine the importance of this protein in
606 the salt tolerance of rice. Here, we have shown that the *sos1* mutant plant displays an
607 exceptional salt-sensitivity that is akin to the Arabidopsis *sos1* mutant phenotype. By
608 contrast to the uniqueness of OsSOS1, the modest salt-sensitivity of the rice *cipk24*
609 mutant relative to *sos1* plants is likely due to functional redundancy. The genetic
610 complement of CIPK-CBL modules in the rice genome comprises 10 CBLs and 33 CIPKs,
611 which is similar to the 10 CBLs and 25 CIPKs known in Arabidopsis (Kolukisaoglu et al.,
612 2004; Gu et al., 2008; Kanwar et al., 2014). Most of the *CIPK* genes of rice showed
613 transcriptional responses to various abiotic stresses including salinity (Xiang et al.,
614 2007; Kanwar et al., 2014). Since transcription of *CIPK8*, the closest homologue to
615 *CIPK24*, was induced by salt (Xiang et al., 2007; Kanwar et al., 2014), a functional
616 relatedness of CIPK8 and CIPK24 appears likely regarding the salt-stress response of
617 rice. Our transcriptomic analysis also flagged CIPK15, CIPK21 and CIPK31 as potential
618 players in the sodicity response of roots based on the greater up-regulation in *sos1*
619 roots compared to that in the wild type (Supplemental Table S5). Indeed, CIPK15 has
620 been reported to increase the salinity tolerance of transgenic rice (Xiang et al., 2007),
621 whereas a loss-of-function *cipk31* mutant was salt sensitive (Piao et al., 2010). There is
622 no reverse genetics data for *CIPK21*, but RT-qPCR confirmed a 21.47-fold up-regulation
623 of *CIPK21* transcripts after 7 d in *sos1* roots (Figure 8). Likely, this large transcriptional
624 induction of *CIPK21* in the *sos1* mutant reflects its major role in Na⁺ homeostasis. Thus,
625 transcriptomic and genetic data, together with the enormous difference between the
626 salt-sensitive phenotype of the *cipk24* and the *sos1* mutants, suggest that several
627 CIPKs may target SOS1 for post-transcriptional activation in rice.

628

629 Recently, a genetic screen for reduced cesium (Cs⁺) accumulation in rice identified a
630 putative loss-of-function allele of *SOS2/CIPK24* as the underlying genetic lesion

631 (Ishikawa et al., 2017). The lower Cs^+ content in the *lcs1/sos2* mutant correlated with
632 reduced expression of K^+ transporters HAK1, HAK5 and AKT1 under low external
633 K^+/Na^+ ratios, suggesting that Cs^+ uptake occurred through root K^+ transporters. Similar
634 to our findings, the *lcs1/sos2* mutant accumulated Na^+ in roots and was moderately
635 salt-sensitive.

636

637 *Entry and redistribution of Na^+*

638

639 The salt tolerance imparted by *SOS1* is so intimately related to cellular tolerance that
640 overexpression of *SOS1* and resistance to NaCl has been used as selection system for
641 rice transformation (Zhu and Wu, 2008). Here, we show that nearly ubiquitous
642 overexpression of *SOS1* driven by the *35S* promoter yielded salt-tolerance that
643 correlated with reduced Na^+ contents in shoots and roots, as should be expected from
644 enhanced efflux of cellular Na^+ (Figure 4). In addition, we have shown that *SOS1* also
645 plays a systemic role by re-distributing Na^+ ions between roots and shoots at the
646 xylem-parenchyma boundary (Figure 6). Rice roots develop Casparian bands and
647 suberin lamellae on the walls of the exodermal and endodermal cell layers that act as
648 physical barriers against the apoplastic flow of water and dissolved minerals
649 (Krishnamurthy et al., 2009). However, lateral roots emerge from the pericycle and
650 disrupt the continuity of the endodermal barrier as they grow towards the cortex and
651 exodermis. This rupture of the apoplastic barrier generates sites through which salts
652 could leak into the main root. The contribution of this 'bypass-flow' to Na^+ uptake in
653 rice is thought to constitute a major component of the salt sensitivity of this crop,
654 particularly at high transpiration rates (Yeo et al., 1987). Opposing this view, later
655 studies found no evidence indicative of Na^+ entry at the sites of secondary root
656 emergence in rice plants although the tissue still remained open to apoplastic flow
657 tracers (Faiyue et al., 2010). Rather, it was concluded that bypass-flow occurred
658 through the lateral roots themselves (Faiyue et al., 2010). Regardless of whether
659 bypass flow occurs through lateral roots or the endodermal rupture points caused by
660 them, our data support the notion that net Na^+ fluxes into and across the root are far
661 from being an uncontrolled process. The combination of the tissue expression pattern
662 of the *SOS1* gene promoter and the profile of Na^+ content along a cross-section of rice

663 roots from wild-type and *sos1* mutant plants submitted to a moderate salinity stress (2
664 d in 50 mM NaCl) indicated that SOS1 prevented, in the first place, the excessive
665 accumulation of Na⁺ in all cell types of the root, and that this protein specifically
666 directed the flow of Na⁺ past the endodermal filter toward the xylem vessels for the
667 acropetal evacuation of Na⁺ out of the root (Figures 5 and 6). Nevertheless, it should
668 be emphasized that SOS1 is primarily a cellular-based Na⁺ efflux system (Shi et al.,
669 2002; Oh et al., 2009) and Na⁺ will accumulate over time in root tissues to greater
670 levels in the *sos1* mutant than that in wild-type plants, which would eventually lead to
671 Na⁺ leaking into shoots by mass flow. Moreover, discontinuities in the root endodermis
672 may also allow Na⁺ export out of roots without a biochemical control by dedicated
673 transporters. In other words, Na⁺ will accumulate in the shoot of the *sos1* mutant as a
674 consequence of uncontrolled uptake by roots even if SOS1 contributes to transporter-
675 mediated Na⁺ loading into the xylem.

676

677 The sensitivity of rice plants to salinity is strongly associated with the accumulation of
678 Na⁺ in the leaves (Coskun et al., 2013). Like *SOS1*, the gene encoding the Na⁺-selective
679 transporter *SKC1/HKT8/HKT1;5* is preferentially expressed in the parenchyma cells
680 surrounding the xylem vessels, where the HKT protein is thought to mediate the
681 recirculation of Na⁺ by unloading Na⁺ from the root xylem (Ren et al., 2005). Our data
682 shows that SOS1 counteracts HKT function by actively exporting Na⁺ from parenchyma
683 cells into the xylem vessels. Thus, the amount of Na⁺ that is eventually delivered to
684 shoots will be determined by the interplay between HKT and SOS1 proteins acting
685 antagonistically. The optimal set-points for Na⁺ root content and the net transfer of
686 Na⁺ from shoots to roots must be commensurate with the ability of shoot tissues to
687 compartmentalize the incoming Na⁺ to avert ion toxicity while achieving full osmotic
688 adjustment (Pardo, 2010). The down-regulation of *SKC1/HKT8/HKT1;5* in salinized *sos1*
689 roots could represent a root-protecting mechanism limiting the back-flow of Na⁺ to
690 roots that are already exposed to high Na⁺ contents. The reciprocally balanced activity
691 of HKT and SOS1 proteins has been evidenced also in wheat. Two loci, termed *Nax1*
692 and *Nax2* encoding proteins HKT1;4 and HKT1;5, respectively, are genetic
693 determinants of enhanced Na⁺ retrieval from the xylem and reduced rates of Na⁺
694 delivery to the shoot (Huang et al., 2006; James et al., 2006; Byrt et al., 2007). Notably,

695 the *Nax* loci also reduced the activity and expression level of the SOS1 exchanger in
696 both root cortical and stele tissues, which contributed further towards reducing the
697 xylem Na⁺ load (Zhu et al., 2015). In Arabidopsis, a functional hierarchy of HKT- and
698 SOS1-dependent transport processes was established by comparing the salt sensitivity
699 of *hkt1* and *sos1-sos3* mutants (Rus et al., 2004). The mutant lacking HKT1 function
700 was sensitive to long-term salinity due to the excessive accumulation of Na⁺ in shoots
701 and the *hkt1* mutation suppressed the NaCl hypersensitivity of the *sos3* mutant by
702 achieving a balanced distribution of Na⁺ that was closer to that of wild-type plants (Rus
703 et al., 2001; Rus et al., 2004). Together, these data demonstrate that a finely regulated
704 interplay between HKT and SOS systems at the xylem parenchyma is a key component
705 of the salt tolerance of vascular plants.

706

707 An unresolved question is the mechanistic basis for the intimate relationship of Na⁺-
708 specific transporters acting at the xylem parenchyma with the K⁺ status of the plant. In
709 rice, both SOS1 and SKC1/HKT8/HKT1;5 selectively transport Na⁺ and yet they affect
710 the accumulation of K⁺ in shoots. Wheat plants carrying the *Nax1* and *Nax2* loci
711 encoding the Na⁺ transporters HKT1;4 and HKT1;5 also had higher rates of K⁺ transport
712 from roots to shoots (James et al., 2006). Root xylem loading is a key step for the
713 delivery of nutrients to the shoot (Drew et al., 1990; Engels and Marschner, 1992;
714 Gaymard et al., 1998). In Arabidopsis, the voltage-dependent K⁺-channel SKOR
715 mediates xylem loading and root-to-shoot translocation of K⁺ (Gaymard et al., 1998).
716 The *SKOR* gene is primarily expressed in pericycle and root xylem parenchyma cells.
717 The *SKOR* channel directs outward K⁺ currents upon membrane depolarization
718 facilitating the release of the K⁺ from the parenchyma cells into the xylem vessels. The
719 Arabidopsis *sos1* mutant was initially described as impaired in K⁺ uptake (Wu et al.,
720 1996), but subsequent research has shown that SOS1 is a highly specific Na⁺/H⁺
721 exchanger with no ability to transport K⁺ (Qiu et al., 2002; Shi et al., 2002; Quintero et
722 al., 2011). Here we show that removal of SOS1 from its preferential expression tissue
723 at the xylem parenchyma cells leads to the accumulation of Na⁺ ions while in transit to
724 the xylem vessels, with the concomitant reduction in K⁺ content in these same cells.
725 The Na⁺/K⁺ ratio in xylem parenchyma cells climbed from 0.49 in the wild type to 9.40
726 in the *sos1* mutant, i.e. a 19-fold change (Figure 6). The coupling of Na⁺ unloading by

727 the xylem-localized HKT transporters with K^+ loading into xylem vessels via
728 depolarization-activated K^+ channels has been hypothesized (Horie et al., 2009). HKT-
729 mediated Na^+ uptake from xylem vessels could induce membrane depolarization of
730 xylem parenchyma cells, which in turn would activate SKOR, the depolarization-
731 activated outward-rectifying K^+ channel mediating K^+ release from xylem parenchyma
732 cells. On the other hand, cytosolic Na^+ has been shown to inhibit the K^+ channel AKT1
733 involved in K^+ uptake by Arabidopsis roots (Qi and Spalding, 2004). Greater cytosolic
734 Na^+ concentration in *sos1* plants resulted in impaired K^+ uptake by roots and poor
735 growth under salt stress. Our data suggest a combined scenario in which the high
736 Na^+/K^+ ratio in cortex cells may interfere with root K^+ uptake and the symplastic
737 movement towards the stele (note that *AKT1* and *AKT2* genes are differentially
738 repressed in *sos1* roots; Supplemental Table S5), while the exorbitant Na^+/K^+ ratio in
739 xylem parenchyma cells of the *sos1* may interfere with K^+ loading into the xylem for
740 nutrient partitioning between shoots and roots. This model may also explain the initial
741 observation that the growth of the Arabidopsis *sos1* mutant was impaired under low
742 K^+ availability. This hypothesis about the mechanistic links between long-distance
743 transport of Na^+ and K^+ nutrition deserves further research.

744

745 *Transcriptome of the sos1 mutant*

746

747 Nitrate (NO_3^-) is the quantitatively most important anion counter-balancing xylem
748 loading of K^+ (Engels and Marschner, 1993). In Arabidopsis, the low-affinity, bi-
749 directional NO_3^- transporter NRT1.5 mediates NO_3^- efflux from pericycle cells to the
750 xylem vessels of Arabidopsis, and SKOR and NRT1.5 cooperate to ensure K^+ delivery to
751 the shoot via xylem (Drechsler et al., 2015). Alternatively, NRT1.5 could load K^+ into the
752 xylem directly (Li et al., 2017; Ragel et al., 2019). A similarly complex relationship is
753 also apparent between Na^+ and K^+ loading into the xylem (Alvarez-Aragon and
754 Rodriguez-Navarro, 2017). Our transcriptomic data of the *sos1* mutant suggests a
755 broad molecular connection between Na^+/K^+ homeostasis and nitrogen metabolism
756 under salinity stress. The biological process, as categorized by GO terms, "Cellular
757 Nitrogen Compound Metabolic Process" was the most severely down-regulated
758 category in the *sos1* mutant under salt relative to that in control roots (FDR= $1.6 \times 10^{-}$

759 ¹⁸). Key proteins involved in nitrate uptake by roots and nitrate assimilation were
760 repressed in the *sos1* mutant. Genes encoding four NRT2-like transporters and two
761 NAR2-like regulators have been identified in the rice genome. The Nitrate Transporter-
762 Activating protein 2.1 (NAR2.1) interacts with the nitrate transporters OsNRT2.1,
763 OsNRT2.2 and OsNRT2.3a to activate the uptake and distribution of nitrate within the
764 plant (Feng et al., 2011). The strong expression of *OsNAR2.1*, *OsNRT2.1* and *OsNRT2.2*
765 in root epidermal cells and that *OsNAR2.1* knockdown greatly impaired both high- and
766 low-affinity nitrate transport systems demonstrated the key role of these proteins in
767 nitrate uptake (Yan et al., 2011). Notably, these three key genes were substantially
768 repressed in *sos1* roots by salinity. The log₂ fold change in transcript abundance for
769 *NAR2.1* (Os02g0595900), *NRT2.1* (Os02g0112100) and *NRT2.2* (Os02g0112600) were -
770 4.24, -3.33 and -2.91, respectively. Similarly, the majority of enzymatic genes involved
771 in the nitrate assimilation by its incorporation to glutamine (nitrate and nitrite
772 reductases, and glutamine synthetases) were down-regulated in salt-treated *sos1*
773 roots. OsCIPK23 (Os07t0150700), the closest rice homologue to the protein kinase
774 CIKP23 that regulates positively nitrate uptake in Arabidopsis (Ho et al., 2009), was
775 also down-regulated in salinized *sos1* roots relative to that in the control (log₂ FC = -
776 1.66), in agreement with a general repression of nitrogen uptake and metabolism.
777 Consequently, the transcriptional repression of these key genes in the *sos1* mutant
778 root should have a profound effect on nitrate uptake from the mineral medium and
779 subsequent nitrogen assimilation, as already indicated by the GO term and MapMan
780 analyses. The physiological implications of this response are presently unclear, but
781 might be related to the mutual stimulation of nitrate and Na⁺ uptakes that has been
782 observed in various species including rice (Kaburagi et al., 2015; Nie et al., 2015; Gao et
783 al., 2016). In Arabidopsis, nitrate-dependent transport systems mediate the uptake
784 and load of Na⁺ into the xylem, and they may constitute a major pathway for the
785 accumulation of Na⁺ in Arabidopsis shoots (Alvarez-Aragon and Rodriguez-Navarro,
786 2017). Thus, repression of nitrate uptake by *sos1* roots could have helped to curtail Na⁺
787 entry and/or distribution.

788

789 An intriguing observation is that the GO term 'Response to Stress' was down-regulated
790 in the *sos1* mutant in the two conditions tested, with and without salt, which in

791 principle meant that the *sos1* mutant was less responsive to stress. A number of genes
792 whose function could be reasonably predicted as being beneficial to overcome salinity
793 stress (*CBL4*, *NHX1*, *NHX2*, and the putative vacuolar malate transporter encoded by
794 Os09g0484900) or genes that are known molecular markers of stress intensity (*SaIT*)
795 were more highly expressed in the *sos1* mutant than that in wild-type roots. These
796 molecular markers and the severely disturbed Na^+/K^+ profiles of *sos1* roots
797 demonstrate that the mutant roots were indeed suffering sodicity stress more
798 intensely than the wild type. Thus, the large proportion of genes down-regulated in
799 the *sos1* mutant might be the consequence of impaired stress detection or an inability
800 to mount a full-fledged response, despite the greater intensity of the stress imposed
801 upon *sos1* roots. Previous studies (Zhu, 2002; Shabala et al., 2005; Ji et al., 2013) have
802 suggested that SOS1 may perform regulatory or sensory functions that may or may not
803 be strictly linked to its function as a Na^+ transporter. The exceedingly high salt-
804 sensitivity of *sos1* mutants of various species (Zhu, 2002; Oh et al., 2009; Olias et al.,
805 2009) and the interaction of the very large C-terminal domain of SOS1 with other
806 stress signaling intermediaries (Katiyar-Agarwal et al., 2006) lend indirect support to
807 this hypothesis. Precedents of this dual function as enzymes and key regulators are the
808 nitrate transporter NTR1.1/CHL1 and the hexokinase HXK1 that serve as sensor
809 proteins of their substrates (Moore et al., 2003; Ho et al., 2009; Bouguyon et al., 2015).
810

811 Alternatively, SOS1 may not be a *bonafide* sensor but serve as a molecular scaffold to
812 facilitate the crosstalk of various signaling pathways needed to mount salt tolerance.
813 The SRO (Similar to RCD-One) proteins are a group of plant-specific proteins that have
814 important functions in stress adaptation and development. In Arabidopsis, SOS1
815 interacts with RCD1, a nucleo-cytoplasmic protein that together with AtSRO1 functions
816 to regulate oxidative stress, hormonal and developmental responses (Katiyar-Agarwal
817 et al., 2006; Jaspers et al., 2010). Moreover, RCD1 Interacts with dehydration-
818 responsive DREB2 proteins and a number of transcription factors belonging to several
819 protein families. The rice genome contains five *SRO* genes (*SRO1a* to *1e*) and
820 transcription of *SRO1c* is highly responsive to various stresses including salinity. As in
821 Arabidopsis, rice *SRO1c* has a negative role in resistance to oxidative stress, which
822 seems to be associated to the repression of ROS-scavenging enzyme genes (You et al.,

2013). Surprisingly, transcriptomic and RT-qPCR data showed a staggering 55- and 168-fold induction of *SRO1c* in *sos1* roots after 4 and 7 d of salt treatment, which runs counterintuitive to the expected output since ROS scavenging is vital to overcome salinity-induced oxidative stress in rice (Hoang et al., 2016) and *OsSRO1c*-overexpressing rice suffers from greater sensitivity to oxidative stress (You et al., 2013). The observed under-representation of DEGs belonging to the GO Biological Process 'Oxidation Reduction' (FDR = 2.3×10^{-6} , Supplemental Table S2) and the GO Molecular Function 'Oxidoreductase Activity' (FDR = 2.6×10^{-5}) in *sos1* roots under salinity are likely related to over-induction of the negative transcription regulator *SRO1c*. Our MapMan analysis of the *sos1* mutant transcriptional response also pointed to a significant enrichment of genes involved in the redox cellular metabolism among the down-regulated DEGs, with 61 down-regulated genes grouped in this category. Detailed analysis of the repressed genes indicates that detoxification enzymes thioredoxins, ascorbate-peroxidases (APXs), catalases and superoxide dismutases were mainly repressed in the mutant relative to that in the wild type. Moreover, further analysis of the mutant transcriptome indicated that the expression of genes grouped in the "drought/salt abiotic stress" category is significantly altered in response to salt (corrected *p*-value = 0.037). The differential expression was surprisingly not due to a higher level of induction of the genes of this category in response to salt, but again to a general repression in the *sos1* mutant relative to the wild-type expression level. Thus, it appears that the lack of *SOS1* triggers a run-away process leading to unsuitable repression of ROS-controlling genes by *OsSRO1c* and exacerbated salt damage. Taken globally, the transcriptomic profile of the *sos1* mutant root is evidence of an as yet undefined but superior regulatory role of *SOS1* in the salinity stress response.

847

848 MATERIALS AND METHODS

849

850 *Plant culture, salinity treatments and chemical analyses*

851

852 Rice seeds were germinated onto wet filter paper for 5 d and then transferred to de-capped and perforated Eppendorf tubes pre-filled with rockwool. Culture was
853 continued in a hydroponics setting with aeration in modified Miyamoto's medium
854

855 (Miyamoto et al., 2001), with weekly transfers to fresh medium. Nutrient medium
856 consisted of 0.09 mM $(\text{NH}_4)_2\text{SO}_4$, 0.05 mM KH_2PO_4 , 0.05 mM KNO_3 , 0.03 mM K_2SO_4 ,
857 0.06 mM $\text{Ca}(\text{NO}_3)_2$, 0.07 mM MgSO_4 , 0.11 mM Fe-EDTA, 4.6 μM H_3BO_3 , 1.8 μM MnSO_4 ,
858 0.3 μM ZnSO_4 , 0.3 μM CuSO_4 , pH 5.7, plus 0.3 μM Na_2MoO_4 . The actual concentrations
859 of K^+ and Na^+ in the medium as measured by Atomic Absorption Spectrophotometry
860 were 0.20 mM K^+ and 0.14 mM Na^+ . The hydroponic containers were placed in a
861 growth chamber set to a daily light/dark cycle of 16/8 h, photosynthetically active
862 radiation 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 30°C/22°C day/night temperature, and 40–60% relative
863 humidity. Salt treatments were given by transferring the plants to fresh medium
864 supplemented with the indicated amounts of NaCl.

865

866 Contrary to Nipponbare plants, seedlings of mutant (L1H and L24H) and congeneric
867 azygous lines (L1A and L24A) showed asynchronous germination and heterogeneous
868 growth rates, which presumably arose from somaclonal variation. Therefore, after one
869 week in hydroponic culture, outlier plants were removed and only those with sizes
870 similar to Nipponbare controls were transferred to fresh media with and without
871 supplemental NaCl.

872

873 For halotolerance tests in experimental paddies in the field, seeds were first
874 germinated in pots with peat. Four-week-old plants of mutant genotypes *sos1* and
875 *cipk24*, their respective congeneric null segregants, and of wild type Nipponbare were
876 then transferred to paddies with clay-loam soil. Plants were arranged in paddies with 5
877 rows (one per genotype) of 10 plants each, to have 4 repetitions (individual paddies) of
878 each growth condition (control and salinized) and 40 plants per genotype and
879 treatment. Genotypes were arranged differently in each individual paddy. Control
880 paddies were irrigated with water from an underground well with a salt content of 0.4
881 g/L. Salinity treatment was imposed by automatic mixing of underground water with a
882 salty brine to give a final concentration of 1.8 g/L of seawater salts in the irrigation
883 water.

884

885 Chlorophyll contents were determined with a Minolta SPAD-502 Chlorophyll Meter
886 and values are given as SPAD units. To measure Na^+ and K^+ contents in tissues, plants

887 exposed to salt stress were separated in shoots and roots and weighed. Roots were
888 washed thoroughly 2–3 times over 2 min with cold distilled water to remove surface-
889 contaminating salts, blotted dry and deep-frozen until use. Dried ground material was
890 autoclaved to extract minerals and the supernatant submitted to Atomic Absorption
891 Spectrophotometry.

892

893 *Rice mutant lines*

894

895 Nomenclature and genes codes are according to the Rice Genome Annotation Project,
896 version 6.1 (<http://rice.plantbiology.msu.edu/>). Putative mutant lines annotated to
897 carry T-DNA or Tos17 insertions in genes *SOS1* (Os12g44360), *SOS2/CIPK24*
898 (Os06g40370) and *SOS3/CBL4* (Os05g45810) in the Nipponbare genetic background
899 were screened for in the public repositories OryGenesDB of Génoplante
900 (<http://orygenesdb.cirad.fr/>), the Rice Functional Genomic Express Database
901 (<http://signal.salk.edu/cgi-bin/RiceGE>), the Rice Tos17 Insertion Mutant Database
902 (<https://tos.nias.affrc.go.jp/>), and the Rice Mutants Database (<http://rmd.ncpgr.cn/>).

903

904 Seeds received from repositories were considered as T2 generation, comprising both
905 heterozygous and homozygous individuals. Plants emerging from these primary T2
906 seed pools were subjected to diagnostic PCR to identify individuals homozygous for
907 the mutagenic insertion based on the presence of the amplicon predicted from the line
908 annotation and by the absence of the wild-type amplicon when oligonucleotides
909 flanking the insertion point were used as primers in the diagnostic PCR. Primers used
910 for diagnostic PCR and RT-PCR are listed in Supplemental Table S6. Congenic null-
911 segregants without the mutagenic insertion (denoted here as azygous plants) were
912 also selected as controls for further experiments to account for possible epigenetic or
913 phenotypic alterations arising during plant transformation and regeneration
914 procedures. Plants selected after diagnostic PCR were used to produce T3 seeds, and
915 T3 plants were subsequently used for RT-PCR and phenotypic analyses.

916

917 *Nucleic acids, plasmids and transformation*

918

919 For RT-PCR analyses, total RNA was isolated from ca. 100 mg of frozen plant leaves
920 with TRIZOL (Invitrogen). Synthesis of cDNA with integrated genomic DNA removal was
921 done from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen).
922 PCR was done with DNA purified as described in (Murray and Thompson, 1980). For
923 sequencing of amplicons and precise identification of insertion points, RT-PCR was
924 performed with Accuzyme (Bioline Reagents Ltd) and amplicons were ligated to vector
925 pCR-Blunt and transformed into *E. coli*. Recombinant plasmids were purified using the
926 Perfectprep Gel Cleanup kit (Eppendorf), diluted at 100 ng/µL in H₂O, and sequenced
927 with M13 Reverse and Forward primers.

928

929 Constructs made to test the function of the rice *SOS1* protein in yeast were done in
930 vector pDR195 carrying the *PMA1* gene promoter. Co-expression of Arabidopsis *SOS2*
931 and *SOS3* was achieved with plasmid pFL32T (Quintero et al., 2002). The ORF encoding
932 the truncated protein OsSOS1Δ970 was obtained by PCR using as template the wild-
933 type cDNA (Martinez-Atienza et al., 2007) and with oligonucleotides that included
934 *BamHI* cutting sites and a stop codon after amino acid L969 of *SOS1*. The resulting
935 amplicon was digested with *BamHI* and inserted in pDR195. Yeast transformation
936 followed the Lithium-Acetate method. The *Saccharomyces cerevisiae* strain AXT3K
937 ($\Delta ena1::HIS3::ena4$, $\Delta nha1::LEU2$, $\Delta nhx1::KanMX4$) has been described elsewhere
938 (Guo et al., 2004). Sodium tolerance tests were performed in the alkali cation-free AP
939 medium (Rodriguez-Navarro and Ramos, 1984) supplemented with 1 mM KCl and with
940 NaCl as indicated in each experiment.

941

942 To determine the expression pattern of *SOS1* a 3.9-kb fragment in the intergenic
943 region between *SOS1* (Os12g44360) and the adjacent gene (Os12g44370) and proximal
944 to the coding region of *SOS1* was amplified by PCR and inserted as a *Sall* and *XbaI*
945 fragment into vector pBIG containing a promoter-less *GUS* reporter gene (Becker,
946 1990). *GUS* chemical staining with X-gal in whole tissues was as described in (Jefferson
947 et al., 1987). To test complementation of the *sos1* mutant, the full-length cDNA of rice
948 *SOS1* was cloned in a pCAMBIA1300-derived vector containing the maize *UBIQUITIN1*
949 gene promoter and the *nopaline synthase* (*NOS*) terminator (Campo et al., 2014). For
950 *SOS1* overexpression, the cDNA was cloned into the vector pCAMBIA2300 under the

951 control of the CaMV 35S promoter and terminator and transformed in Nipponbare
952 plants. Rice transformation by *Agrobacterium tumefaciens* EHA105 has been described
953 (Campo et al., 2014).

954

955 *Measurement of Na⁺/H⁺ exchange in membrane vesicles*

956

957 Plasma membrane vesicles were isolated from 2-month-old rice roots from 24-h
958 salinized (50 mM NaCl) plants using two-phase partitioning as described by Qiu et al.,
959 (2003). All steps were carried out at 4°C or on ice. Roots were homogenized in buffer
960 containing 0.33 M sucrose, 10% (w/v) glycerol, 0.2% (w/v) BSA, 5 mM EDTA, 5 mM
961 DTT, 5 mM ascorbate, 0.2% (w/v) casein, 0.6% (w/v) polyvinylpyrrolidone (PVP-10), 1
962 mM PMSF, 1 µg/mL pepstatin A, and 50 mM HEPES-KOH (pH 7.5). Eight milliliters of
963 homogenization buffer were used per gram of tissue. The homogenate was filtered
964 through two layers of Miracloth (Merck) and centrifuged at 10,000 *g* for 10 min. The
965 supernatant then was centrifuged for 50 min at 100,000 *g* to obtain a microsomal
966 pellet, that was resuspended in a buffer containing 0.33 M sucrose, 3 mM KCl, 0.1 mM
967 EDTA, 1 mM DTT, 1 mM PMSF, 1 µg/mL pepstatin A, and 5 mM potassium phosphate
968 (pH 7.8). The suspension was added to a phase mixture to obtain a phase system
969 consisting of 6.2% (w/w) Dextran T-500 and 6.2% (w/w) polyethylene glycol 3350 in 5
970 mM potassium phosphate (pH 7.8), 0.33 M sucrose, 3 mM KCl. Phase separation was
971 facilitated by centrifugation at 1,000 *g*. The upper phase was then repartitioned twice
972 in this system. The final upper phase was collected, diluted with suspension buffer
973 [0.33 M sucrose, 10% (w/v) glycerol, 0.1 mM EDTA, 2mM DTT, 1 µg/mL pepstatin A, 20
974 mM HEPES-KOH (pH 7.5)], and centrifuged for 45 min at 100,000 *g*. The resulting pellet
975 was collected and resuspended with the above-described suspension buffer containing
976 1 mM EDTA, frozen in liquid nitrogen and stored at -80°C until use. Before membrane
977 isolation plants were treated for 24 h with 50 mM NaCl in Miyamoto's medium to
978 induce SOS1 activity. Vesicles purified from non-treated plants produced very low
979 rates of Na⁺/H⁺ antiport activity, in agreement with previous reports (Qiu et al., 2003).

980

981 The measurement of Na⁺/H⁺ proton exchange was as described by Qiu et al. (2003),
982 except that fluorescence quenching of 9-amino-6-chloro-2-methoxy-acridine (ACMA)

983 was used to monitor the formation and dissipation of pH gradients. Fluorescence was
984 recorded with a Hitachi fluorescence spectrophotometer (FL-2500) in a thermostated
985 cell (25°C) at excitation and emission wavelengths of 415 and 485 nm, respectively
986 (slit-width, 10 nm). Purified plasma membranes (50 µg of protein) were added to a
987 buffer containing 250 mM mannitol, 10 mM bistris-propane-2-(N-morpholine)-
988 ethanesulphonic acid (BTP-MES) (pH 6.8), 100 mM KCl, 3 mM MgSO₄ and 1 µM ACMA
989 (1 mL final volume). The ΔpH was generated with the activity of the plasma membrane
990 H⁺-ATPase and recorded as fluorescence quenching after ATP addition (2.5 mM ATP,
991 pH 6.8). The Na⁺-induced dissipation of ΔpH was initiated after adding 100 mM NaCl
992 into the cuvette, and recorded as the recovery of fluorescence. Exchange rates are
993 calculated as fluorescence recovery relative to that prior to ATP addition (ΔF/F_{max})
994 per minute and milligram of protein.

995

996 *Microarray analysis*

997

998 Control and *sos1* seedlings were grown hydroponically in Miyamoto's medium for 3
999 weeks in environmentally controlled conditions (12 h of light at 25°C and ca. 450 µmol
1000 quanta m⁻² s⁻¹, and 12 h darkness at 21°C; relative humidity ca. 65%). Salt stress was
1001 imposed by transferring to fresh nutrient solution containing 75 mM NaCl for 4 d.
1002 Control plants received fresh nutrient solution without NaCl. No signs of plant
1003 deterioration were observed at the end of the salinity treatment. Total RNA was
1004 extracted from whole roots pooled from five plants per genotype and treatment. The
1005 quality and concentration of the RNA were determined using the Agilent 2100
1006 Bioanalyzer and samples with an RNA Integrity Number (RIN) less than 6 were
1007 discarded. Three on-chip technical replicates per biological sample were analyzed.
1008 Gene expression profiling was performed by microarray hybridization using a custom
1009 rice transcriptomic array (Oryzon Genomics, Barcelona, Spain) containing 3 x 29450
1010 probes (3 technical replicates per probe) representing 20750 genes. Microarray
1011 construction, probe labeling, and hybridization conditions were as in (Campo et al.,
1012 2014). Results were normalized using self-to-self comparison Nipponbare vs
1013 Nipponbare, and fold change was calculated as log₂ of the ratio of relative expression
1014 of *sos1* (sample, Smp) versus wild-type (control, Ctr) plants grown in identical

1015 conditions. Only genes for which replicate spots showed differential expression of -0.5
1016 $> \log_2(\text{Smp}/\text{Ctr}) > 0.5$ and P value < 0.05 were considered as differentially expressed.
1017 Bootstrap analysis with Significance Analysis of Microarrays was used to identify
1018 differentially expressed genes using a cut-off of 2 (Tusher et al., 2001). Significance
1019 Analysis of Microarrays calculates the fold change and significance of differences in
1020 expression. Data sets for microarray analyses have been deposited in the National
1021 Center for Biotechnology Information Gene Expression Omnibus with accession
1022 number GSE129844.

1023

1024 Differential expression of a select number of DEGs was confirmed by reverse-
1025 transcription quantitative PCR (RT-qPCR). Three-week-old control and *sos1* seedlings
1026 were treated with 75 mM NaCl in Miyamoto's medium for 4 and 7 d. Total RNA was
1027 extracted from roots and 500 ng were used to synthesize the cDNA using the
1028 Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany). Quantitative real-time
1029 PCR (qRT-PCR) assays were performed using the CFX Connect Real-Time PCR Detection
1030 System (Bio-Rad). The PCR mixture (total volume of 20 μ l) was composed of 5 μ l of
1031 diluted cDNA, 10 μ l iTaq Universal SYBR Green supermix (2x), 0.3 μ M of each primer
1032 and H₂O. The specific oligonucleotides used are listed in the Supplemental Table S6.
1033 Thermal cycling consisted of 95°C for 3 min, followed by 40 cycles of 10 sec at 95°C, 15
1034 sec at 58°C, and 15 sec at 72°C. A melting curve was generated to check the specificity
1035 of the amplified fragment. The efficiency of all primers under these conditions was 85–
1036 100% for all samples tested. Rice *UBIQUITIN5* was used as a house-keeping gene in
1037 expression analysis. Relative gene expression was determined using the $2^{-\Delta\Delta\text{CT}}$
1038 method (Livak and Schmittgen, 2001). Threshold cycle values were determined using
1039 ICYCLER Q software (Bio-Rad).

1040

1041 *Statistical analyses*

1042

1043 Numerical data was analyzed with IBM SPSS Statistics v.21 (Armonk, NY, USA) using a
1044 completely randomized design model. Means and variances were compared by One-
1045 Way Analysis of Variance (ANOVA) to find significant differences using the Fisher's
1046 Least Significant Differences test at $p < 0.01$ level, unless stated otherwise.

1047

1048 **ACCESSION NUMBERS**

1049 Sequence data from this article can be found in the Rice Genome Annotation Project
1050 (<http://rice.plantbiology.msu.edu/>) and UniProt (<https://www.uniprot.org>) databases
1051 under accession numbers *SOS1* (LOC_Os12g44360, Q5ICN3), *CIPK24/SOS2*
1052 (LOC_Os06g40370, Q69Q47) and *CBL4/SOS3* (LOC_Os05g45810, Q75KU4).

1053

1054 **SUPPLEMENTAL MATERIAL**

1055

1056 **Supplemental Figure S1.** Molecular characterization of rice lines AKFG08 and AKFB09.

1057

1058 **Supplemental Figure S2.** Molecular characterization of rice line NC2588.

1059

1060 **Supplemental Figure S3.** Molecular characterization of rice line AUJE11.

1061

1062 **Supplemental Figure S4.** Molecular characterization of the rice line AVIB03.

1063

1064 **Supplemental Figure S5.** Na⁺/H⁺ exchange activity in plasma membrane vesicles.

1065

1066 **Supplemental Figure S6.** Molecular characterization of the rice lines AMHC11 and
1067 AMHC12.

1068

1069 **Supplemental Figure S7.** Salinity tolerance test in experimental paddies.

1070

1071 **Supplemental Figure S8.** Complementation of the *sos1* mutant.

1072

1073 **Supplemental Figure S9.** MapMan analysis of the metabolic and cellular responses of
1074 *sos1* roots to salt.

1075

1076 **Supplemental Table S1.** Differentially expressed genes (DEGs) in the *sos1* mutant roots
1077 when compared to the control line (Excel file).

1078

1079 **Supplemental Table S2.** Gene Ontology (GO) analysis of total DEGs identified in the
1080 salt-stressed or control-cultivated roots (Excel file).

1081

1082 **Supplemental Table S3.** Top 25 enriched GO categories (Excel file).

1083

1084 **Supplemental Table S4.** MapMap analysis of DEGs identified in the salt-stressed *sos1*
1085 roots (Excel file)

1086

1087 **Supplemental Table S5.** Differential expression levels of select genes involved in Na⁺
1088 and K⁺ transport processes (Excel file).

1089

1090

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1092

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1100

1101

1102 **TABLES**

1103

1104 **Table 1.** Insertion points (nt, nucleotide) are expressed relative to the ATG initiation
1105 codon in the genomic sequences, corresponding to nucleotides 27508321 in *SOS1*,
1106 24043690 in *CIPK24* and 26535878 in *CBL4*. When confirmed by sequencing of the PCR
1107 amplicon containing the mutagenic insertion, the actual insertion point is also given;
1108 otherwise it is noted as not determined (n.d.). OTL, Oryza Tag Line database; NIAS,
1109 National Institute of Agrobiological Sciences, Japan.

1110

Gene	Source	Line code	Mutagen	Annotated insertion	Confirmed insertion
SOS1	NIAS	NC2588	Tos17	Intron 5, nt 1228	Intron 5, nt 1228
SOS1	OTL	AVIB03	Tos17	Intron 19, nt 9798	Intron 19, nt 9802
SOS1	OTL	ARBF06	Tos17	Exon 20, nt 9951	No insertion
SOS1	OTL	AKFB09	Tos17	Exon 21, nt 11232	Intron 20, nt 11096
SOS1	OTL	AKFG08	Tos17	Exon 21, nt 11312	Intron 20, nt 11096
SOS1	OTL	AFNB04	Tos17	Exon 21, nt 11402	No insertion
SOS1	OTL	AUJE11	Tos17	Intron 21, nt 11410	Intron 21, nt n.d.
CIPK24	OTL	AMHC11			
		AMHC12	T-DNA	Exon 1, nt -84	Exon 1, nt -84
		AMHE12			
CBL4	OTL	AJBC12	T-DNA	3'-UTR	n.d.

1111

1112

1113 **FIGURE LEGENDS**

1114

1115 **Figure 1. Functionality of truncated SOS1 protein.**

1116 A, Diagram of functional domains of SOS1. The junction domain is the C-terminal
1117 portion of SOS1 that has sequence homology with NHX8 and no known functional role;
1118 further downstream is the regulatory domain that is specific and characteristic of
1119 SOS1-like proteins that has been dissected in three functional modules: the activation
1120 domain with sequence similarities to cyclic-nucleotide binding site (amino acids 740–
1121 820), the autoinhibitory domain (977–1023) and the CIPK24 phosphorylation site (Ser-
1122 1135) that counteracts autoinhibition (Quintero et al., 2011). The arrowhead marks
1123 the truncation point after K767 in the mutant protein. B, RT-PCR of *SOS1* mRNA of
1124 wild-type Nipponbare plants (WT) and homozygous plants of line AVIB03 (*sos1*), with
1125 primers S1-E19-F and S1-E20-R annealing at exons 19 and 20. Detached leaves were
1126 treated (salt) or not (unt) with 150 mM NaCl for 24 h. Insertion of Tos17 at the
1127 intervening intron 19 prevented the amplification in the *sos1* mutant. As control, the
1128 cDNA of *SOS1* was used as template in the PCR reaction (cDNA); C- indicates a mock
1129 reaction without template. C, Truncated SOS1 proteins of rice were expressed in the
1130 yeast strain AXT3K, with and without the co-expression of the Arabidopsis SOS2–SOS3
1131 kinase complex, and compared to the wild-type SOS1. Decimal dilutions of saturated
1132 liquid cultures in selective media were plated in AP medium with 1 mM KCl (1K)
1133 supplemented with the indicated millimolar concentrations of NaCl.

1134

1135 **Figure 2. Growth parameters of *sos1* mutant in salt.**

1136 Five-d-old seedlings of wild type, *sos1* mutant, and a congenic segregant without the
1137 *sos1* mutation, were transferred to hydroponic culture in Miyamoto's medium
1138 supplemented with the indicated amounts of NaCl. A, Depicted are three
1139 representative plants per genotype of wild type (WT) and *sos1* mutant at 5 and 10 d
1140 after transfer to 25, 50 and 75 mM NaCl. B, Fresh weight (FW) and dry weight (DW) of
1141 shoot and roots, the chlorophyll content (SPAD units), and the shoot and root Na⁺
1142 content of wild type (black columns), *sos1* (white) and the congenic azygous control
1143 (grey) before and after one week in 50 mM NaCl. The chlorophyll content at 14 d is
1144 also given. Plants after 2 weeks growth in salt were not further processed because

1145 roots of the *sos1* mutant were severely damaged. Values are the mean and SD of five
1146 plants per genotype. Different letters indicate significantly different values relative to
1147 that in the wild type determined by One-Way ANOVA ($a= p<0.01$).

1148

1149 **Figure 3. Plant growth in experimental paddies.**

1150 A, Representative plants of *sos1* (L1H), *SOS1* congenic segregant (L1A) and wild type
1151 Nipponbare (WT) genotypes grown for 2 months in experimental paddies irrigated
1152 with underground water (left) or supplemented with salt (right). Salt contents in the
1153 irrigation water are indicated on top. B, Na^+ and K^+ content in shoots of control (white
1154 columns) and salinized plants (grey columns). Genotypes are labeled as in (A). Plotted
1155 are the mean and SD of five plants per genotype and condition after 30 d of saline
1156 treatment. Different letters indicate significantly different values at $p<0.001$
1157 determined by One-Way ANOVA.

1158

1159 **Figure 4. Complementation of *sos1* mutant.**

1160 Ten-d-old seedlings of wild type, *sos1* mutant, and the complementation line C11 were
1161 transferred to hydroponic culture in Miyamoto's medium supplemented with the
1162 indicated amounts of NaCl. Plants were harvested two weeks later. A, Depicted are
1163 representative plants of genotypes Nipponbare (N), the *sos1* mutant (S) and line C11
1164 (C). B, Dry weight and Na^+ contents in shoots and roots of plants harvested after 7 d of
1165 the salinity treatment. Represented are the mean values and SD of wild type (black
1166 columns), *sos1* (white) and complementation line C11 (grey) of four plants per
1167 genotype and growth condition. Letters indicate significantly different values relative
1168 to that in the wild type determined by One-Way ANOVA ($a= p<0.01$, $b= p<0.05$).

1169

1170 **Figure 5. Salt tolerance imparted by *SOS1***

1171 Seven-d-old seedlings of two independent Nipponbare transgenic lines expressing
1172 *35S:SOS1* were cultivated alongside control seedlings transformed with an empty
1173 vector (V) in the absence or presence of 25 and 50 mM NaCl for 4 weeks. A,
1174 Representative control (V) and transgenic plants (*SOS1*) are depicted. B, Survival rate
1175 of plants after the completion of the experiment. C, Dry weight (DW) and Na^+ content
1176 (as percentage of DW) of plants surviving the 25-mM NaCl treatment ($n=6$). Letters

1177 indicate significantly different values relative to that in the wild type determined by
1178 One-Way ANOVA (a= $p<0.01$, b= $p<0.05$, c= $p<0.1$).

1179

1180 **Figure 6. Tissue expression pattern of *SOS1*.**

1181 A to K, Representative images of staining patterns resulting from GUS expression
1182 under the control of the *SOS1* gene promoter. A and B, Preferential expression around
1183 the root vascular bundle. C, Root cross-section. D and E, Lack of staining in
1184 undifferentiated tissues near the root cap and in root hairs. F to H, Staining indicates
1185 that expression of *SOS1* correlates with the differentiation of vascular bundles in
1186 secondary roots. I, Scattered expression around the vasculature of the coleoptile. J,
1187 Vascular bundles of the flag leaf. K, Strong expression in leaf xylem parenchyma.
1188 Arrowheads point to non-stained stomata. The scale bars represent 100 μm .

1189

1190 **Figure 7. Mineral content analysis by SEM-EDX.**

1191 A, Representative SEM cross section of root with labelled arrows indicating the cell
1192 types whose elemental composition was analyzed by EDX. Scale bar is 0.1 mm. B,
1193 Percentage of total counts of Na^+ and K^+ , measured in 4–5 cells of indicated cell types
1194 from 2 plants of each genotype. Plants were treated with 50 mM NaCl for 2 d. Shown
1195 are the mean and SD values. Letters indicate significantly different values relative to
1196 that in the wild type for each cellular type determined by One-Way ANOVA (a= $p<0.01$;
1197 b= $p<0.05$). C, Na/K ratios of net content values given in (B). The experiment was
1198 repeated twice with similar results.

1199

1200 **Figure 8. Summary of Differentially Expressed Genes**

1201 A, Four-way Venn diagram indicating the number of up-regulated and down-regulated
1202 genes found in the comparison between the *sos1* rice mutant and the wild type
1203 Nipponbare in the non-stress (left; Control: 0 mM) and salt-stress (right; Salt: 75 mM)
1204 treated roots. (B) Three-way Venn diagram indicating the number of significant GO
1205 terms from the gene-ontology analysis of the up-regulated, down-regulated and total
1206 DEGs found in the *sos1* rice mutant and the wild type Nipponbare in salt-stressed
1207 roots.

1208

1209 **Figure 9. Quantitation of expression levels of genes relevant to salinity tolerance.** RT-
1210 qPCR analysis was performed using the $2^{-\Delta\Delta CT}$ method and the gene expression data
1211 normalized for the quantity of *UBQ5* gene transcript before (0) and 4- and 7-d after
1212 salt treatment with 75 mM NaCl. All y axes are mean values of normalized expression
1213 levels \pm SD. All x axes are time of treatment and genotype.
1214
1215

1216

1217

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