

# A dual role for the OsK5.2 ion channel in stomatal movements and K+ loading into xylem sap.

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19	One sentence summary: The rice K+ channel OsK5.2 is necessary for rapid stomatal closure
20	and contributes to shoot K+ supply from the root, thus highlighting the differing functional
21	distribution of Shaker channels compared to Arabidopsis
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24	
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#### 54 ABSTRACT

55 The roles of potassium channels from the Shaker family in stomatal movements have been investigated by reverse genetics analyses in Arabidopsis, but corresponding information is 56 lacking outside this model species. Rice and other cereals possess stomata that are more complex 57 than those of Arabidopsis. We examined the role of the outward Shaker  $K^+$  channel gene OsK5.2. 58 59 Expression of the OsK5.2 gene (GUS reporter strategy) was observed in the whole stomatal 60 complex (guard cells and subsidiary cells), in the root vasculature and root cortex. In stomata, 61 loss of OsK5.2 functional expression resulted in lack of time-dependent outward potassium 62 currents in guard cells, higher rates of water loss through transpiration, and severe slowdown of 63 stomatal closure. In line with the expression of OsK5.2 in the plant vasculature, mutant plants displayed a reduced K<sup>+</sup> translocation from the root system towards the leaves via the xylem. The 64 comparison between rice and Arabidopsis show that despite the strong conservation of Shaker 65 66 family in plants, substantial differences can exist between the physiological roles of seemingly orthologous genes, as xylem loading depends on SKOR and stomatal closure on GORK in 67 68 Arabidopsis, whereas both functions are executed by the single OsK5.2 Shaker in rice.

69

#### 70 INTRODUCTION

71 Since a waxy cuticle covers outer leaf tissues, water vapor diffusion into the atmosphere 72 occurs mainly through the stomatal pores at the leaf surface. The size of the stomatal aperture is 73 tightly regulated to optimize gas exchanges between the leaf inner tissues and the atmosphere, 74 including CO<sub>2</sub> intake for photosynthesis and water loss by transpiration (Lawson and Blatt, 75 2014). This is achieved by fine tuning of the turgor pressure of the two guard cells which 76 surround the stomatal pore, and involves a complex coordinated activity of transport systems at the guard cell plasma membrane and vacuolar membrane (Hedrich, 2012; Chen et al., 2012; Hills 77 78 et al., 2012; Kollist et al., 2014). This control also affects long distance transport of mineral nutrients from the roots, which take up these nutrients, to the aerial parts, to support plant growth(Marschner et al., 1996).

Potassium ion (K<sup>+</sup>), as a major inorganic constituent of the plant cells and the most abundant 81 cation in the cytosol, is an essential macronutrient for growth and development. It is involved in 82 83 various functions including electrical neutralization of negative charges, control of cell 84 membrane polarization and osmoregulation (Clarkson and Hanson, 1980; Leigh and Wyn Jones, 1984).  $K^+$  is thus the main cation absorbed by the roots and circulating within the plant at the 85 86 cellular or long distance levels. In guard cells, it is well known as a major contributor, with Cl, 87 NO<sub>3</sub><sup>-</sup> and malate, to the osmolarity (Raschke and Schnabl, 1978; Willner and Fricker, 1996). 88 Stomatal opening is initiated by activation of plasma membrane proton pumps in guard cells, which promotes K<sup>+</sup> influx through voltage-gated inward K<sup>+</sup> channels, as well as anion uptake 89 through H<sup>+</sup>-anion symporters (Blatt, 1987a; Schroeder et al., 1987; Roelfsema and Prins, 1997; 90 91 Talbott and Zeiger, 1998; Guo et al., 2003; Jezek and Blatt, 2017). Conversely, stomatal closure 92 requires inhibition of proton pumping at the guard cell membrane and activation of both anion channels and voltage-gated outward K<sup>+</sup> channels. 93

The molecular mechanisms responsible for inward and outward K<sup>+</sup> fluxes across the plasma 94 95 membrane have been extensively investigated in Arabidopsis. Shaker channel subunits, present as 96 a 9-member family in Arabidopsis, have been shown to form the major pathways for these fluxes 97 throughout the plant (Véry and Sentenac, 2003). In the Arabidopsis model species, four genes 98 encoding Shaker channel subunits have been identified as playing a major role in root to shoot K<sup>+</sup> 99 translocation and in stomatal movements. The SKOR subunit, which is expressed in root 100 pericycle and xylem parenchyma, forms outwardly-rectifying channels involved in  $K^+$  secretion 101 into the xylem sap (Gaymard et al., 1998). In stomata, the inward Shaker channel subunits KAT1 and KAT2 are involved in guard cell  $K^+$  uptake, and the outward Shaker channel GORK 102 mediates guard cell K<sup>+</sup> release (Ache et al., 2000; Pilot et al., 2001; Szyroki et al., 2001; Hosy et 103 104 al., 2003; Lebaudy et al., 2008). Whereas these Shaker subunits have been deeply characterized 105 in Arabidopsis and the Shaker family, as a whole, can be considered as the best characterized 106 family of plant membrane transport systems, little information at the molecular genetic level is 107 yet available on this family outside Arabidopsis.

108 The stomatal complex in rice (*Oryza sativa* L.), the present model cereal, is very different 109 from that of Arabidopsis (Itoh et al., 2005; Franks and Farquhar, 2007; Roelfsema and Hedrich, 110 2009). In rice like in other cereals, it comprises two subsidiary cells in addition to the two guard 111 cells. Substantial differences may thus exist between rice and Arabidopsis in stomatal functioning 112 (Mumm et al., 2011). Rice possesses two putative outward Shaker channel subunits (Pilot et al., 113 2003; Véry et al., 2014), named OsK5.1 (or OsSKOR) and OsK5.2 (or OsGORK) (Pilot et al., 114 2003; Kim et al., 2015). OsK5.1 was reported to be mainly expressed in root vasculature, just as 115 SKOR in Arabidopsis (Kim et al., 2015). In contrast, OsK5.2 was found to be expressed both in 116 roots and shoots (Kim et al., 2015). The expression pattern of this gene at the tissue level has, 117 however, not been described. Here, we investigate the expression pattern and role of this rice 118 outward Shaker gene. OsK5.2 is shown to play two important roles in rice plants: it mediates  $K^+$ 119 translocation into the xylem sap towards the shoots, and it is involved in K<sup>+</sup> release from guard 120 cells and stomatal movements.

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122

#### 123 **RESULTS**

#### 124 The outward Shaker channel subfamily in Arabidopsis and rice

The Shaker group 5, which consists of the outward Shaker subunits, usually comprises 2 or 3 members in the plant genomes analyzed so far (Pilot et al., 2003; Véry et al., 2014). Figure 1 illustrates the phylogenetic relationships between the members of the Shaker group 5 present in Arabidopsis and in 3 monocots, rice, maize (*Zea mays*) and *Brachypodium distachyon*. Based on this phylogenetic tree, the rice OsK5.1 and OsK5.2 subunits appeared to belong to two different subgroups within the Shaker group 5. The distance between these subgroups was larger than that between GORK and SKOR from Arabidopsis.

132

#### 133 Expression pattern of OsK5.2

134 Transgenic rice plants expressing GUS under the control of the OsK5.2 promoter region 135 (Fig. 2A) were generated to investigate the expression of this Shaker gene (Fig. 2, B-J). GUS 136 staining revealed that OsK5.2 was expressed in vascular tissue of root (Fig. 2, E-G), like OsK5.1 137 (Kim et al., 2015), as well as in the shoot vascular tissue (Fig. 2, B, D). In addition, the OsK5.2 138 promoter was active in root cortical cells (except at the root tip) (Fig. 2, E-G). Weak GUS 139 staining was also observed in flowers, in the pollen sacs and grains (Fig. 2, H-J). Analysis of 140 GUS staining in leaf epidermis revealed expression of OsK5.2 in stomata, the staining being 141 stronger in guard cells than in subsidiary cells (Fig. 2, B-D).

142 The following experiments were aimed at investigating the role of OsK5.2 in K<sup>+</sup> translocation 143 towards the shoots and in stomatal movements.

144

#### 145 **Production of** *osk5.2* **loss-of-function mutant plants**

Genotyping experiments performed on the progeny of the mutant lines ASJA08 and ASHF06 (Supplemental Figure 1A) allowed us to identify *osk5.2* homozygous mutant plants (*Tos17* insertion alleles), as well as plants without insertion in the *OsK5.2* gene, hereafter named wildtype controls. The offspring of the selected plants (*osk5.2* mutants and wild-type controls) was used for in planta experiments. RT-PCR experiments on total RNA prepared from the *osk5.2* 



**Figure 1.** Phylogenetic analysis of outward Shaker channels in monocot species and *Arabidopsis thaliana*. The unrooted phylogenetic trees were generated with PhyML software (http://www.atgc-montpellier.fr/phyml/binaries.php) using the maximum-likelihood method and 1000 bootstrap replicates in Seaview application (http://doua.prabi.fr/software/seaview). The scale bar corresponds to a distance of 10 changes per 100 amino acid positions. The protein (GenBank) accession numbers are given in Supplemental Table S2. At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Os, *Oryza sativa* (rice); Zm, *Zea mays* (maize).

151 mutants and the wild type control plants confirmed the loss of function of OsK5.2 in the selected

152 osk5.2 homozygous mutant lines (Supplemental Figure 1B).

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**Figure 2.** Expression pattern of *OsK5.2* outward Shaker channel gene in rice. A, *Promoter::GUS* construct (promoter region size: 2339 bp). B to J, GUS activity in transgenic rice plants, revealed by an overnight incubation in the presence of X-Gluc: leaf tissues (B), stomata (C), leaf cross-section (D), root (E), root tip (F), root cross-section (G), flower (H), anther (I), and pollen (J).

## 154 Impaired K<sup>+</sup> transport into the xylem sap and K<sup>+</sup> accumulation in shoots in *osk5.2* mutant

155 plants

156 K<sup>+</sup> was assayed in roots and shoots of 5 week-old plants hydroponically grown on standard 157 Yoshida medium. Comparison of osk5.2 mutant plants with the corresponding wild type plants revealed that the absence of OsK5.2 functional expression did not affect K<sup>+</sup> accumulation in 158 159 roots, but resulted in significantly reduced  $K^+$  contents (by ~25%) in shoots (Fig. 3, A and B, left panels). The K<sup>+</sup> concentration measurements, in xylem sap samples which were collected after 160 161 leaf excision, revealed lower values, by 30 to 40%, in the mutant plants (Fig. 3, A and B, right panels). Thus, the whole set of data indicated that the absence of OsK5.2 functional expression 162 resulted in a decrease in K<sup>+</sup> translocation from roots to shoots via the xylem sap. 163

164

# Increased transpirational water loss due to impaired stomatal movements in *osk5.2* mutant plants

Water consumption (essentially due to transpirational water loss) was monitored in *osk5.2* mutant plants and the corresponding wild-type control plants hydroponically grown for 5 weeks on Yoshida medium using a multi-potometer (Supplemental Figure S2). Both mutant lines displayed larger steady state rates of transpirational water loss than the corresponding wild-type plants in the light, by about 25% (Fig. 4).

172 The transpiration rate is dependent on stomatal aperture (Pallas Jr, 1965; Hosy et al., 2003). 173 Light to dark transitions were used to provoke stomatal closure. The decrease in transpiration rate 174 when light was switched off was slower in the mutant plants than in the corresponding wild-type 175 control plants (Fig. 5, A and B, top panels), revealing a slower rate of dark-induced stomatal 176 closure in absence of OsK5.2 functional expression. Fitting the decrease in transpiration rates 177 with exponential functions yielded a time constant of approximately 10 min for the wild-type 178 control plants, and of 20-30 min for the mutants from the two osk5.2 lines (Fig. 5, A and B, 179 bottom panels).

The kinetics of the light-induced increase in transpiration rate was also studied (Fig. 6). In wild type plants, when light was switched on, the transpiration rate displayed a rapid increase, which was followed by a transient decrease. The *osk5.2* mutant plants also displayed a rapid increase in transpiration but the kinetics was slightly slower than in the wild type plants (Fig. 6, A and B, bottom panels), and no following transient decrease in transpiration rate was observed (Fig. 6).



**Figure 3.** Tissue K<sup>+</sup> contents and xylem sap K<sup>+</sup> concentration in wild-type and *osk5.2* mutant plants. Five week-old plants were grown in standard Yoshida hydroponic solution. A and B, Root and shoot K<sup>+</sup> contents (left) and xylem sap K<sup>+</sup> concentration (right) in wild-type control (black bars) and *osk5.2* mutant (white bars) plants issued from the initial AJSA08 (A) or ASHF06 (B) lines. Assayed xylem sap exudates were collected in wild-type control and *osk5.2* mutant plants after 1-h exudation in the case of the plants issued from the AJSA08 line, and 2-h exudation in the case of the plants issued from the ASHF06 line. Data are means  $\pm$  SE (*n* = 3 or 4 for wild-type control plants, and 4 or 5 for *osk5.2* mutant plants in A, and *n* = 4 or 6 for wild-type control plants, and 5 or 6 for *osk5.2* mutant plants in B). An asterisk indicates that the corresponding difference in K<sup>+</sup> content or concentration between the wild-type control and *osk5.2* mutant plants were statistically significant (Student's t test, P < 0.05).

186

## 187 Absence of Shaker-type outwardly rectifying K<sup>+</sup> channel activity in guard cells of *osk5.2*

188 mutant plants



**Figure 4.** Steady-state transpiration rates in wild-type control and *osk5.2* mutant plants. Five week-old plants issued from the initial ASJA08 line (above) or ASHF06 line (below) were used for steady-state transpiration rate analyses in dark (*ca.* 5 h after light was switched off) and in light (*ca.* 3 h after light was switched on) conditions. The steady-state transpiration rate (in mL/cm<sup>2</sup>/h) was determined by dividing the average speed of water loss at steady-state (mean of 3 values) by the total area of the plant aerial parts. Black and white bars represent wild-type control and *osk5.2* mutant plants, respectively. Data are means  $\pm$  SE; n = 6 for wild-type control and 13 for *osk5.2* mutant plants in the case of the plants issued from the ASJA08 line, and n = 7 for wild-type control and 9 for *osk5.2* plant plants in the case of the plants issued from the ASJF06 line. An asterisk indicates that the corresponding difference between the wild-type control and mutant plants was statistically significant (Student's t test, P < 0.05).

The consequences of OsK5.2 loss of function mutation on the K<sup>+</sup> conductance of the guard cell membrane were analyzed *in planta*. Guard cells from two-week-old plants were impaled with double-barreled electrodes (Blatt, 1990; Thiel et al., 1992; Roelfsema et al., 2001) and inward



**Figure 5.** Kinetics of the decrease in transpiration rate upon dark-induced stomatal closure in wild-type and *osk5.2* mutant plants. A and B, Kinetics (top) and time constant (bottom) of the dark-induced decrease in transpiration rate in wild-type control ( $\bigcirc$ ,  $\blacksquare$ ) and *osk5.2* mutant ( $\bigcirc$ ,  $\square$ ) plants issued from the initial ASJA08 (A) or ASHF06 (B) lines in standard Yoshida hydroponic solution. The light was switched off after 30 min of recording under light condition as indicated by the change from white to black in the bar below the graph. Transpiration rates were normalized by dividing absolute transpiration rates by the steady-state transpiration rate in light condition, and are presented as percentages (%). Time constants were derived by fitting the experimental dark-induced transpiration rate decrease kinetics with an exponential function (least-squares fitting). Black and white bars correspond to wild-type control and *osk5.2* mutant plants, respectively. Data are means  $\pm$  SE; n = 6 for wild-type control and 13 for *osk5.2* mutant plants in A, and n = 7 for wild-type control and 9 for *osk5.2* mutant plants in B. The asterisks indicate that the corresponding differences between the wild-type control and mutant plants were statistically significant (Student's t test, P < 0.05).

and outward currents through the membrane were elicited by voltage clamp pulses (Fig. 7A). In
wild-type guard cells, depolarizing voltage pulses elicited slowly activating outward currents
(Fig. 7B, left panel, and Fig. 7C), clearly reminiscent of those mediated by the outwardly-



**Figure 6.** Kinetics of the increase in transpiration rate upon light-induced stomatal opening after a long period of darkness in wild-type and *osk5.2* mutant plants. A and B, Kinetics of light-induced increase in transpiration rate (top) and time of the corresponding half-increase in transpiration rate (bottom) in wild-type control ( $\bullet$ ,  $\blacksquare$ ) and *osk5.2* mutant (O,  $\square$ ) plants from the initial ASJA08 (A) and ASHF06 (B) lines in standard Yoshida hydroponic solution. The change from black to white in the bar below the curves indicates the time at which light was switched on after the dark period. The normalized transpiration rate (%) was determined as described in Figure 5. Times of half light-induced increase in transpiration rate were derived from the fit of the experimental data up to the end of the initial rapid phase of light-induced transpiration rate increase to a sigmoidal equation (least-squares fitting). Data are means  $\pm$  SE; n = 6 for wild-type control and 13 for *osk5.2* mutant plants in A, and n = 7 for wild-type control and 9 for *osk5.2* mutant plants in B). An asterisk indicates that the corresponding difference between the wild-type control and mutant plants in time of half-increase in transpiration rate was statistically significant (Student's t test, P < 0.05).

- rectifying K<sup>+</sup> channels from the Shaker family (Gaymard et al., 1998; Hosy et al., 2003; Hedrich,
- 196 2012; Véry et al., 2014). Slowly activating outward currents were lacking in *osk5.2* mutant plants
- 197 (Fig. 7, D and E). In contrast, the inward conductance appeared to be very similar in osk5.2



**Figure 7.** Voltage-clamp experiments with guard cells in intact rice plants. A, Voltage-clamp protocols used to record either outward (left) or inward (right) currents in rice guard cells. Starting at a holding value of -100 mV, the voltage was shifted in 20 mV steps to either +20 mV, or -220 mV. B and D, Representative outward (left) and inward (right) current traces recorded in guard cells from ASJA08 line wild-type control (B) or *osk5.2* mutant (D) plants. C and E, Average current-voltage relationships obtained in wild-type control (C) or mutant (E) guard cells. Data are means  $\pm$  SE (n = 6 in C, and n = 4 in E).

mutant and wild type guard cells (Fig. 7, B and D, right panels). This suggested that, although *in planta* voltage-clamp analyses can give rise to experimental drawbacks (Blatt, 1987b; Roelfsema
et al., 2001), comparison between wild type and mutant guard cell currents was valid in our

201 conditions, and thus that OsK5.2 encodes slowly-activating outwardly rectifying K<sup>+</sup> channels in 202 rice guard cells. It was also observed that the absence of OsK5.2 channel activity caused a 203 positive shift of the free running membrane potential (cross point with X-axis in the current-204 voltage relationship), by about 50 mV (Fig. 7, C and E).

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206

#### 207 DISCUSSION

#### 208 Comparison of the expression patterns of the outward Shaker genes in rice and Arabidopsis

In rice, as in Arabidopsis, the outward Shaker channel group comprises two members. However, based on the phylogenetic tree displayed in Figure 1, OsK5.1 and OsK5.2 cannot be simply considered as the orthologs of the Arabidopsis outward Shaker channels SKOR and GORK. OsK5.2 and OsK5.1 belong to two separate subgroups within the group of outward Shaker channels from cereal plant species (Fig. 1).

214 In Arabidopsis, SKOR has been reported to be essentially expressed in the root vasculature 215 (Gaymard et al. 1998), and GORK in root periphery cells (except in the root apex) and in stomata 216 (Ache et al., 2000; Ivashikina et al., 2001). The expression pattern of OsK5.1 in rice is 217 reminiscent of that of SKOR in Arabidopsis, with strong expression in root vasculature (Kim et 218 al., 2015). In contrast, OsK5.2 displays high expression levels in both the root vasculature (Fig. 2 219 E-G) and the stomata (Fig. 2 B-D), and thus its expression pattern clearly encompasses the 220 patterns reported for both SKOR and GORK in Arabidopsis (Gaymard et al., 1998; Ache et al., 221 2000; Ivashikina et al., 2001). It is not yet known whether the expression patterns of OsK5.1 and 222 OsK5.2 are shared by genes belonging to the same outward Shaker subgroups from other 223 monocots. The expression pattern of only one maize gene from OsK5.2 subgroup has been 224 partially characterized so far, by qRT-PCR analyses (Büchsenschütz et al., 2005). Like OsK5.2, 225 this maize outward Shaker gene is expressed in both stomata and roots, and thus its expression 226 pattern may be similar to that of OsK5.2.

227

#### 228 Functional properties of OsK5.2 channels

229 Members of Shaker group 5 have been cloned in several plant species and successfully 230 expressed in heterologous systems (*Xenopus* oocyte essentially) for electrophysiological analysis 231 of their functional properties (Véry et al., 2014). They all behave as slowly-activating outwardly-232 rectifying K<sup>+</sup>-selective channels. However, so far, all the channels for which such functional 233 analyses have been reported belong to dicotyledonous species. We attempted to express OsK5.2 234 in *Xenopus* oocytes, but no exogenous current in the oocytes was detected using the classical 235 two-electrode voltage-clamp technique (Véry et al., 1995; Tounsi et al., 2016). This might indicate that these outward K<sup>+</sup> channels have to be co-expressed with regulatory protein partners
to be active at the cell membrane, as reported for number of members of the inwardly rectifying
Shaker channel group 1 from dicots (Xu et al., 2006; Honsbein et al., 2009; Cuéllar et al., 2010;
Cuéllar et al., 2013) and monocots (Boscari et al., 2009; Geiger et al., 2009).

240 Our voltage-clamp measurements with intact guard cells from wild type plants and osk5.2 241 mutants (Fig. 7) indicate that OsK5.2 displays functional features reminiscent of group 5 Shaker 242 channels as determined upon expression in heterologous systems (Ache et al., 2000; Langer et al., 243 2002; Johansson et al., 2006; Sano et al., 2007), especially a strong outward rectification, a slow 244 activation upon membrane depolarization and slow deactivation of the so-called tail currents 245 when the membrane is repolarized. Finally, the fact that these Shaker-type outward currents were 246 no longer observed in guard cells from the osk5.2 mutants indicates that OsK5.2 is the major 247 contributor to the expression of such a channel activity at the guard cell membrane.

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#### 249 Comparison of the roles of OsK5.2 and SKOR in K<sup>+</sup> translocation towards the shoots

Both ASJA08 and ASHF06 *osk5.2* loss-of-function mutations caused reduced leaf K<sup>+</sup> contents, by 21 to 24% (Fig. 3). Taking into account that *OsK5.2* displays expression in root vascular tissues, the simplest explanation is that *OsK5.2*-encoded channel activity contributes to K<sup>+</sup> secretion into the xylem sap in roots, like SKOR in Arabidopsis, and thereby in K<sup>+</sup> translocation towards the shoots. This hypothesis received support from the assay of K<sup>+</sup> in xylem sap exudates from excised roots (Fig. 3), which revealed a marked concentration decrease (by >30%) in the *osk5.2* loss-of-function mutants.

257 In Arabidopsis, the absence of SKOR channel activity due to a loss-of-function mutation has been shown to lead to a decrease in the concentration of  $K^+$  in the xylem sap by about 40%, and 258 259 to a decrease in the accumulation of K<sup>+</sup> in shoots by about 50% (Gaymard et al., 1998). Thus, the contribution of SKOR to the control of K<sup>+</sup> translocation and accumulation in leaves seems a bit 260 261 higher than that of OsK5.2 in rice. Since SKOR is the only outward Shaker channel gene expressed in the root stele, K<sup>+</sup>-permeable channels belonging to other families and/or other types 262 263 of transport systems (e.g., from the KUP/HAK family; Han et al., 2016), have to significantly contribute to K<sup>+</sup> secretion into the xylem sap in Arabidopsis. The situation may be different in 264 265 rice, where both outward Shaker channel genes OsK5.1 and OsK5.2 are expressed in the plant vasculature (Fig. 2; Kim et al., 2015). In other words, besides OsK5.2, another outward Shaker channel, OsK5.1, could contribute to K<sup>+</sup> translocation towards the shoots.

268

## 269 Comparison of the roles of OsK5.2 and GORK in control of stomatal aperture and 270 transpirational water loss

271 Altogether, the present data indicate that OsK5.2 enhances the rate of stomatal closure by mediating  $K^+$  efflux through the guard cell membrane, just as its counterpart GORK in 272 273 Arabidopsis (Hosy et al., 2003). The kinetics of dark-induced decrease in transpiration rate 274 indicated that stomata can close in absence of this outwardly rectifying Shaker-type channel 275 activity in rice, like in Arabidopsis, but the closure kinetics is slowed down. It is likely that poorly selective cation channels (Hedrich, 2012) substitute for Shaker channels as K<sup>+</sup> efflux 276 systems, but stomatal closure is then less efficient. The defect in stomatal closure in osk5.2 277 278 mutant plants is also likely to result from the large difference in free running membrane potential, 279 by approximately 50 mV, between osk5.2 and wild type guard cells (Fig. 7). Indeed, in osk5.2 280 guard cells, the free running voltage across the plasma membrane was shifted towards the 281 reversal potential of anion channels, which was approximately +25 mV (as in Kollist et al., 282 2014). Thus, the driving force for anion efflux (difference between the free running membrane 283 potential and reversal potential of anion channels) was reduced in osk5.2 guard cells. This is 284 expected to cause a decrease in anion efflux, and thus in the rate of stomatal closure.

285 Besides its impact on the rate of dark-induced stomatal closure and steady-state stomatal aperture in darkness, the absence of Shaker-type outward K<sup>+</sup> channel activity in rice is also likely 286 287 to result in larger steady-state stomatal aperture in light, based on the observation that the steady 288 state transpiration rate was increased (Fig. 4). This would mean that OsK5.2 outwardly-rectifying 289 channel activity displays an inhibitory effect on steady-state stomatal aperture in light. The fact that the leaf  $K^+$  content was lower in the *osk5.2* mutant plants (Fig. 3) is likely to lead to a 290 291 decrease in turgor of the leaf cells. A decrease in the turgor of epidermal and subsidiary cells 292 more important than that of guard cells could result in reduced back-pressure on stomatal guard 293 cells and, thus, in increased steady state stomatal aperture in the light. The impact of OsK5.2 294 activity on steady-state transpiration rate in light could be understood within the framework of 295 this latter hypothesis.

296

#### 297 CONCLUSION

298 By controlling the transpirational water loss at leaf surface, stomata also regulate the flux of 299 water from roots to shoots within the xylem vasculature, and thereby contribute to the regulation 300 of nutrient ion translocation towards the shoots. In Arabidopsis, various ion transport systems 301 play a role in both ion translocation into the xylem sap towards the shoots and ion transport 302 across the guard cell membrane and control of stomatal aperture. This is the case, for example, of 303 the anion channel SLAH3 (Geiger et al., 2011; Cubero-Font et al., 2016), the nitrate transporter 304 NRT1.1 (Guo et al., 2003; Wang et al., 2012) and the phosphate transporter PHO1 (Hamburger et al., 2002; Zimmerli et al., 2012). In contrast, regarding K<sup>+</sup>, some kind of specialization has 305 306 occurred within the Shaker group 5 in Arabidopsis since K<sup>+</sup> release from guard cells for stomatal closure has been strictly assigned to GORK, and K<sup>+</sup> secretion into the xylem sap to SKOR. In 307 rice, OsK5.2 contributes to both  $K^+$  release by guard cells for stomatal closure and  $K^+$ 308 309 translocation towards the shoots. The physiological significance of such evolutionary differences 310 would be interesting to clarify. The organization and the roles of the Shaker family are strongly 311 conserved in plants, allowing one to assume functional equivalence between orthologs, but the 312 present data clearly indicate that the biological variability deserves to be investigated since 313 differences between plant species in the use of this gene family are likely to play a role in major 314 physiological functions and in adaptation to environmental constraints.

315

#### 316 MATERIALS AND METHODS

#### 317 Plant material and growth conditions

Rice Nipponbare cultivar (*Oryza sativa* L. var. Nipponbare) was used in all the experiments. For transpiration analysis, seeds were germinated in Petri dishes containing deionized water. One-week-old plants were then transferred onto Yoshida hydroponic solution in 10 L containers (0.7 mM KNO<sub>3</sub>, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.6 mM MgSO<sub>4</sub>, 60  $\mu$ M Na<sub>2</sub>FeEDTA, 45  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 20  $\mu$ M MnSO<sub>4</sub>, 1.6  $\mu$ M CuSO<sub>4</sub>, 1.4  $\mu$ M ZnSO<sub>4</sub>, and 0.3  $\mu$ M (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, pH adjusted to 5.5). Plants were grown in growth chambers (day/night 12 h/12 h photoperiod, 28 °C/25 °C, photon flux density 500  $\mu$ mol.m<sup>-2</sup>.s<sup>-1</sup>, 80% relative humidity). 325

#### 326 Construction of reporter gene constructs and transgenic plants

327 OsK5.2 promoter region (2339 bp) was amplified by PCR from rice Nipponbare genomic 328 DNA using the primers listed in table S1. The PCR product was then cloned into pGemTeasy 329 vector for sequencing before sub-cloning into destination vector. The promoter was then 330 transferred upstream the GUS reporter gene in pCAMBIA1301 vector, where it replaced 331 CAMV35S promoter. The resulting plasmid was introduced into Agrobacterium tumefaciens 332 EHA105 strain for rice transformation, as described by Sallaud et al. (2002). Briefly, dehulked 333 rice seeds were rinsed in ethanol 70% for 90 seconds and treated with disinfection solution 334 (50 g/L of sodium hypochlorite) for 30 minutes. After sterilization, the seeds were transferred to a 335 callus induction medium leading to calluses development from the scutellum region. Rice grains 336 with developing calluses were incubated with bacteria suspension for 90 to 180 seconds and co-337 cultured during 3 days. After the co-culture period, developing calluses were washed with 338 carbenicillin (500 mg/L), separated, divided into small pieces and transferred to shoot induction 339 medium containing hygromycin for transgenic tissue selection. Shoots regenerated from calluses 340 were separated and transferred to rooting medium. Four-week-old plantlets were transferred to 341 soil and grown in greenhouse for multiplication and selection of homozygous plants in the next 342 generation using antibiotics leaf painting method (Cotsaftis et al., 2002).

343

#### 344 Histochemical analysis of GUS activity

345 Leaf samples of 4 week-old plants and flowers were collected from homozygous plants grown 346 in a greenhouse. Root samples were collected from 1 week-old plants grown in standard Yoshida 347 hydroponic medium. All samples were submerged in GUS solution (50 mM Phosphate Buffer 348 pH 7, 2.5 mM ferricyanide, 2.5 mM ferrocyanide, 0.05% v/v Triton X100 and 1 mM X-Gluc) 349 contained in a 24-well plate, the leaves having been previously quickly divided into 1x1 cm 350 pieces. The plate was placed under vacuum for 30 minutes to facilitate the penetration of GUS 351 solution into the tissues, then the samples were incubated overnight at 37°C. After the incubation 352 step, green samples were washed with 70% ethanol solution to remove chlorophyll. Leaf samples were furthermore treated overnight with chloral hydrate (2.5 mg/L in 30% glycerol) before being
observed under microscope (BH2, Olympus) for GUS activity detection.

Thin (8-μm thick) sections were prepared, using a RM 2165 microtome (Leica, Germany),
from GUS-stained fixed samples (pre-fixation in 50 mM phosphate buffer pH 7, 1.5%
formaldehyde, 0.05% Triton X100; fixation in 75 mM phosphate buffer, 2% paraformaldehyde,
0.5% glutaraldehyde) embedded in Technovit 7100 resin (Kulzer, Wehrheim, Germany).

359

#### 360 Selection of KO mutant and wild type control plants

361 Tos17 insertion mutant lines (cv Nipponbare background) displaying insertion in OsK5.2 gene 362 were ordered from OTL collection (CIRAD-Genoscope, France; www.oryzatagline.cirad.fr; 363 Piffanelli et al., 2007). Homozygous mutant plants as well as "wild-type" plants for OsK5.2 gene 364 were selected by PCR on genomic DNA extracted from plantlets issued from received seeds, 365 using Phire® Plant Direct PCR Kit (Thermo Fischer Scientific) and specific primers hybridizing 366 on the target gene and on the Tos17. Total RNA of 2 plants selected in the progeny of a 367 homozygous mutant, or of a plant displaying a wild-type genotype for OsK5.2 (hereafter 368 generically named wild-type control plants), were isolated using RNeasy kit (QIAGEN) and used 369 as templates to synthesize total cDNA. PCR experiments, using primers hybridizing downstream 370 from the Tos17 insertion sites (listed in Supplemental Table S1), allowed us to check the 371 presence (in the wild-type control plants) or absence (in the osk5.2 mutants) of OsK5.2 372 transcripts.

373

#### 374 Voltage-clamp recordings on intact plant guard cells

Two-electrode voltage-clamp experiments were performed on intact guard cells (Roelfsema et al., 2001; Mumm et al., 2011) of intact plants grown on standard Yoshida hydroponic solution for two weeks. A plant (wild type control or homozygous *osk5.2* mutant) was transferred into a 15 mL tube (Falcon type) containing about 5 mL of standard Yoshida solution, the tube being inclined so that the root system was fully immerged in the solution. Outside of the tube, the youngest fully developed leaf was attached to a Plexiglas block with double-sided adhesive tape, the plant remaining intact. The whole plant, attached with its leaf to the plexiglass, was carefully 382 mounted onto the table of an upright microscope (Axoiskop 2FS, Zeiss). About 0.5 to 1 mL of 383 bath solution (5 mM potassium citrate, 5 mM KCl, 0.1 mM CaCl<sub>2</sub>, pH 5.0) was placed between 384 the leaf and the water immersion objective (Achroplan 40/0.80W, Zeiss), used to visualize the 385 stomata. Stomatal opening was induced by light provided by the microscope lamp. Guard cells 386 were impaled using double-barreled electrodes prepared from 1-mm-diameter glass capillaries 387 (1403547, Hilgenberg, Germany), filled with 300 mM KCl. A glass capillary filled with 300 mM 388 KCl and plugged with 300 mM KCl in 2% agarose was placed in the drop of solution between 389 the leaf and the objective and served as a reference electrode. The series resistance present 390 between leaf apoplast and reference electrode was estimated by measuring the resistance between 391 two drops of bath solution placed on the leaf surface, at a distance of approximately 1 cm. This 392 approach revealed a series resistance (which was not different between wild type and osk5.2 393 mutant plants) of  $1.3 \pm 0.1$  (n=5) MOhm at the abaxial leaf face. With guard cell currents lower 394 than 1 nA (Fig. 7), such a series resistance leads to an overestimation of the applied voltage of 395 less than 2 mV. The potential difference between the bath solution and guard cell apoplast 396 ("surface" potential) was  $-10 \pm 2$  mV (n = 6). Because the series resistance and potential 397 difference between the external solution and the apoplast appeared as low, no attempt was made 398 to correct their impact on current-voltage plots. The Win WCP V5.1.6 software (J. Dempster, 399 Strathclyde University, Glasgow, UK) was used to setup the protocol and record applied voltage-400 and current traces, using an ITC-16 interface (Instrutech Corp., Elmont, NY) and a GeneClamp 401 500 (Axon Instruments) amplifier.

402

#### 403 Analysis of stomatal movements induced by light or darkness

Wild type control and homozygous *osk5.2* mutant plants were grown in parallel in standard Yoshida hydroponic medium for five weeks. One day before the experiment, plants were transferred into a multi-potometer device (Supplemental Figure S2), the root system bathed in standard Yoshida hydroponic solution. On the day of the experiment, transpiration rates started to be recorded after 2 h under light condition. A dark treatment was imposed after 30 minutes of recording and was maintained for 4 h before switching back to light condition.

410

#### 411 Analysis of K<sup>+</sup> content in tissues and xylem sap

Root and shoot K<sup>+</sup> contents were assayed in plants grown as described above for the analysis of stomatal movements, i.e., hydroponic conditions for 5 weeks on standard Yoshida solution. After excision, roots and shoots were weighed, dried (60°C during 2 days) and weighed again. Ions were extracted from the tissues with 0.1 N HCl during 2 days. K<sup>+</sup> content in the extracts was determined by flame spectrophotometry (SpectrAA 220FS, Varian).

417  $K^+$  concentration in xylem sap was analyzed in 5 week-old plants grown in standard Yoshida 418 hydroponic solution. Excision at 1 cm above the crown allowed collection of the xylem sap. 419 Exudation duration before collection (identical in control and *osk5.2*mutant plants) lasted for 1 to 420 2 h. Collected samples were diluted in 0.1 N HCl and the concentration of K<sup>+</sup> was determined 421 like that of tissue extracts.

422

#### 423 Accession numbers

424 Sequence data for *OsK5.2* Shaker gene can be found in the GenBank library under accession
425 number Os06g0250600.

426

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432

#### 433 FIGURE LEGENDS

Figure 1. Phylogenetic analysis of outward Shaker channels in monocot species and *Arabidopsis thaliana*. The unrooted phylogenetic trees were generated with PhyML software
(http://www.atgc-montpellier.fr/phyml/binaries.php) using the maximum-likelihood method and
1000 bootstrap replicates in Seaview application (http://doua.prabi.fr/software/seaview). The

scale bar corresponds to a distance of 10 changes per 100 amino acid positions. The protein
(GenBank) accession numbers are given in Supplemental Table S2. At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Os, *Oryza sativa* (rice); Zm, *Zea mays* (maize).

**Figure 2.** Expression pattern of *OsK5.2* outward Shaker channel gene in rice. A, *Promoter::GUS* construct (promoter region size: 2339 bp). B to J, GUS activity in transgenic rice plants, revealed by an overnight incubation in the presence of X-Gluc: leaf tissues (B), stomata (C), leaf cross-section (D), root (E), root tip (F), root cross-section (G), flower (H), anther (I), and pollen (J).

**Figure 3.** Tissue  $K^+$  contents and xylem sap  $K^+$  concentration in wild-type and *osk5.2* mutant 445 446 plants. Five week-old plants were grown in standard Yoshida hydroponic solution. A and B, Root and shoot  $K^+$  contents (left) and xylem sap  $K^+$  concentration (right) in wild-type control (black 447 448 bars) and osk5.2 mutant (white bars) plants issued from the initial AJSA08 (A) or ASHF06 (B) 449 lines. Assayed xylem sap exudates were collected in wild-type control and osk5.2 mutant plants 450 after 1-h exudation in the case of the plants issued from the AJSA08 line, and 2-h exudation in 451 the case of the plants issued from the ASHF06 line. Data are means  $\pm$  SE (n = 3 or 4 for wildtype control plants, and 4 or 5 for *osk5.2* mutant plants in A, and n = 4 or 6 for wild-type control 452 plants, and 5 or 6 for osk5.2 mutant plants in B). An asterisk indicates that the corresponding 453 454 difference in K<sup>+</sup> content or concentration between the wild-type control and *osk5.2* mutant plants were statistically significant (Student's t test, P < 0.05). 455

456 Figure 4. Steady-state transpiration rates in wild-type control and osk5.2 mutant plants. Five 457 week-old plants issued from the initial ASJA08 line (above) or ASHF06 line (below) were used 458 for steady-state transpiration rate analyses in dark (ca. 5 h after light was switched off) and in 459 light (ca. 3 h after light was switched on) conditions. The steady-state transpiration rate (in 460  $mL/cm^{2}/h$ ) was determined by dividing the average speed of water loss at steady-state (mean of 3 461 values) by the total area of the plant aerial parts. Black and white bars represent wild-type control and osk5.2 mutant plants, respectively. Data are means  $\pm$  SE; n = 6 for wild-type control and 13 462 463 for osk5.2 mutant plants in the case of the plants issued from the ASJA08 line, and n = 7 for 464 wild-type control and 9 for osk5.2 plant plants in the case of the plants issued from the ASHF06 465 line. An asterisk indicates that the corresponding difference between the wild-type control and 466 mutant plants was statistically significant (Student's t test, P < 0.05).

467 Figure 5. Kinetics of the decrease in transpiration rate upon dark-induced stomatal closure in 468 wild-type and osk5.2 mutant plants. A and B, Kinetics (top) and time constant (bottom) of the dark-induced decrease in transpiration rate in wild-type control ( $\bullet$ ,  $\bullet$ ) and *osk5.2* mutant ( $\circ$ ,  $\circ$ ) 469 470 plants issued from the initial ASJA08 (A) or ASHF06 (B) lines in standard Yoshida hydroponic 471 solution. The light was switched off after 30 min of recording under light condition as indicated 472 by the change from white to black in the bar below the graph. Transpiration rates were 473 normalized by dividing absolute transpiration rates by the steady-state transpiration rate in light 474 condition, and are presented as percentages (%). Time constants were derived by fitting the 475 experimental dark-induced transpiration rate decrease kinetics with an exponential function 476 (least-squares fitting). Black and white bars correspond to wild-type control and osk5.2 mutant 477 plants, respectively. Data are means  $\pm$  SE; n = 6 for wild-type control and 13 for osk5.2 mutant plants in A, and n = 7 for wild-type control and 9 for *osk5.2* mutant plants in B. The asterisks 478 479 indicate that the corresponding differences between the wild-type control and mutant plants were 480 statistically significant (Student's t test, P < 0.05).

481 Figure 6. Kinetics of the increase in transpiration rate upon light-induced stomatal opening after 482 a long period of darkness in wild-type and osk5.2 mutant plants. A and B, Kinetics of light-483 induced increase in transpiration rate (top) and time of the corresponding half-increase in 484 transpiration rate (bottom) in wild-type control ( $\bullet$ ,  $\blacksquare$ ) and osk5.2 mutant ( $\circ$ ,  $\Box$ ) plants from the 485 initial ASJA08 (A) and ASHF06 (B) lines in standard Yoshida hydroponic solution. The change 486 from black to white in the bar below the curves indicates the time at which light was switched on 487 after the dark period. The normalized transpiration rate (%) was determined as described in 488 Figure 5. Times of half light-induced increase in transpiration rate were derived from the fit of 489 the experimental data up to the end of the initial rapid phase of light-induced transpiration rate 490 increase to a sigmoidal equation (least-squares fitting). Data are means  $\pm$  SE; n = 6 for wild-type 491 control and 13 for osk5.2 mutant plants in A, and n = 7 for wild-type control and 9 for osk5.2492 mutant plants in B). An asterisk indicates that the corresponding difference between the wild-type 493 control and mutant plants in time of half-increase in transpiration rate was statistically significant 494 (Student's t test, P < 0.05).

495 **Figure 7.** Voltage-clamp experiments with guard cells in intact rice plants. A, Voltage-clamp 496 protocols used to record either outward (left) or inward (right) currents in rice guard cells.

- 497 Starting at a holding value of -100 mV, the voltage was shifted in 20 mV steps to either +20 mV,
- 498 or -220 mV. B and D, Representative outward (left) and inward (right) current traces recorded in
- 499 guard cells from ASJA08 line wild-type control (B) or osk5.2 mutant (D) plants. C and E,
- 500 Average current-voltage relationships obtained in wild-type control (C) or mutant (E) guard cells.
- 501 Data are means  $\pm$  SE (n = 6 in C, and n = 4 in E).
- 502

#### 503 Supplemental Material

504 Supplemental Figure S1. Selection of knock-out mutant plants for *OsK5.2* gene.

Supplemental Figure S2. Potometer device. The multi-potometer system used for plant
 transpiration rate monitoring.

Supplemental Table S1. Primer sequences used for cloning, genotyping, and verification of KO
 *osk5.2* mutant plants.

Supplemental Table S2. GenBank accession numbers of Shaker polypeptides present in
 phylogenetic trees

511

**Supplemental Figure S1.** Selection of knock-out mutant plants for *OsK5.2* gene. A, *Tos17* insertion sites in the *osk5.2* mutant ASJA08 (FST accession CU316012) and ASHF06 (FST accession CU316066) lines. B, Lack of *OsK5.2* transcripts in homozygous *osk5.2* mutants issued from ASJA08 and ASHF06 lines. RT-PCR experiments were performed for *OsK5.2* and *eEF1-β* (internal control) using total mRNA isolated from roots of 5 week-old wild-type control (WT) or homozygous mutant (ho) plants issued from each insertion line.

518

519 **Supplemental Figure S2.** Potometer device. The multi-potometer system used for plant 520 transpiration rate monitoring consisted of twenty 50 mL syringes (Terumo corporation, 521 Philippines) vertically fixed into a polystyrene plaque laid over an empty container, and 522 connected to graduated 1 mL serological plastic pipettes ("1 mL Stripette", Costar Corporation, 523 Cambridge, MA) via thin silicone tubes. A camera allowed to record the water level in the 524 pipettes over time. Four to five week-old plants were inserted through the black rubber plunger of 525 the syringes via a 4 mm hole at the plunger center, and sealed by dental polyvinylsiloxane-based 526 elastomer (Coltène® PRESIDENT light body). After polymerization of the elastomer, the 527 plungers were placed to the syringes filled with experimental solution (Yoshida medium). The 528 pipettes and connecting tubes were filled with the same solution, ensuring that no air bubble was 529 present in the system. The transpiration rate was calculated from the decrease in level of the 530 solution in the pipettes recorded over time.

531

Supplemental Table S1. Primer sequences used for cloning, genotyping, and verification of KO
 *osk5.2* mutant plants.

534

535 Supplemental Table S2. GenBank accession numbers of Shaker polypeptides present in
 536 phylogenetic trees

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