A Dual Role for the OsK5.2 Ion Channel in Stomatal Movements and K^+ Loading into Xylem Sap[1]

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The roles of potassium channels from the Shaker family in stomatal movements have been investigated by reverse genetics analyses in Arabidopsis (*Arabidopsis thaliana*), but corresponding information is lacking outside this model species. Rice (*Oryza sativa*) and other cereals possess stomata that are more complex than those of Arabidopsis. We examined the role of the outward Shaker K^+ channel gene *OsK5.2*. Expression of the *OsK5.2* gene (*GUS* reporter strategy) was observed in the whole stomatal complex (guard cells and subsidiary cells), root vasculature, and root cortex. In stomata, loss of *OsK5.2* functional expression resulted in lack of time-dependent outward potassium currents in guard cells, higher rates of water loss through transpiration, and severe slowdown of stomatal closure. In line with the expression of OsK5.2 in the plant vasculature, mutant plants displayed a reduced K^+ translocation from the root system toward the leaves via the xylem. The comparison between rice and Arabidopsis show that despite the strong conservation of Shaker 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 Arabidopsis, whereas both functions are executed by the single OsK5.2 Shaker in rice.

Since a waxy cuticle covers outer leaf tissues, water vapor diffusion into the atmosphere occurs mainly through the stomatal pores at the leaf surface. The size of the stomatal aperture is tightly regulated to optimize gas exchanges between the leaf inner tissues and the atmosphere, including CO_2 intake for photosynthesis and water loss by transpiration (Lawson and Blatt, 2014). This is achieved by fine tuning of the turgor pressure of the two guard cells that 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 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^+), as a major inorganic constituent of the plant cells and the most abundant cation in the cytosol, is an essential macronutrient for growth and development. It is involved in various functions, including electrical neutralization of negative charges, control of cell 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 cellular or long-distance levels. In guard cells, it is well known as a major contributor, with Cl^-, NO_3^- and malate, to the osmolarity (Raschke and Schnabl, 1978; Willmer and Fricker, 1996). Stomatal opening is initiated by activation of plasma membrane proton pumps in guard cells, which promotes K^+ influx through voltage-gated inward K^+ channels, as well as anion uptake through H^+-anion symporters (Blatt, 1987a; Schroeder et al., 1987; Roelfsema and Prins, 1997; Talbott and Zeiger, 1998; Guo et al., 2003; Jezek and Blatt, 2017). Conversely, stomatal closure requires inhibition of proton pumping at the guard cell membrane and activation of both anion channels and voltage-gated outward K^+ channels.

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A.-A.V., H.S., and T.H.N. conceived the original research plans; A.-A.V., E.G., and M.R.G.R. supervised the experiments; D.M., M.R.G.R., S.H., and T.H.N. performed most of the experiments; C.C. and R.M. provided technical assistance to T.H.N.; T.H.N. designed the experiments and analyzed the data; A.-A.V., H.S., and T.H.N. conceived the project and wrote the article with contributions of all the authors; A.-A.V. and H.S. supervised the writing.

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The molecular mechanisms responsible for inward and outward K^+ fluxes across the plasma membrane have been extensively investigated in Arabidopsis (*Arabidopsis thaliana*). Shaker channel subunits, present as a nine-member family in Arabidopsis, have been shown to form the major pathways for these fluxes throughout the plant (Véry and Sentenac, 2003). In the Arabidopsis model species, four genes encoding Shaker channel subunits have been identified as playing a major role in root to shoot K^+ translocation and in stomatal movements. The SKOR subunit, which is expressed in root pericycle and xylem parenchyma, forms outwardly rectifying channels involved in K^+ secretion 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 mediates guard cell K^+ release (Ache et al., 2000; Pilot et al., 2001; Szyroki et al., 2001; Hosy et al., 2003; Lebaudy et al., 2008). Whereas these Shaker subunits have been deeply characterized in Arabidopsis, and the Shaker family, as a whole, can be considered as the best characterized family of plant membrane transport systems, little information at the molecular genetic level is yet available on this family outside Arabidopsis.

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

RESULTS

The Outward Shaker Channel Subfamily in Arabidopsis and Rice

The Shaker group 5, which consists of the outward Shaker subunits, usually comprises two or three 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 three 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.

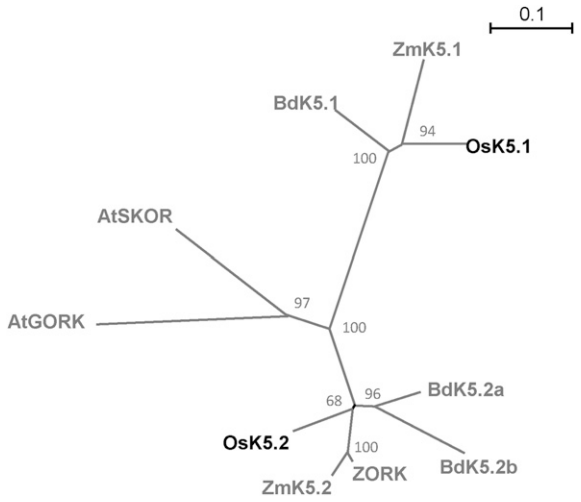

Figure 1. Phylogenetic analysis of outward Shaker channels in monocot species and Arabidopsis. The unrooted phylogenetic tree was generated with PhyML software (http://www.atgc-montpellier.fr/phyml/binaries.php) using the maximum likelihood method and 1,000 bootstrap replicates in Seaview application (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).

Expression Pattern of *OsK5.2*

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

The following experiments were aimed at investigating the role of *OsK5.2* in K^+ translocation toward the shoots and in stomatal movements.

Production of *osk5.2* Loss-of-Function Mutant Plants

Genotyping experiments performed on the progeny of the mutant lines ASJA08 and ASHF06 (Supplemental

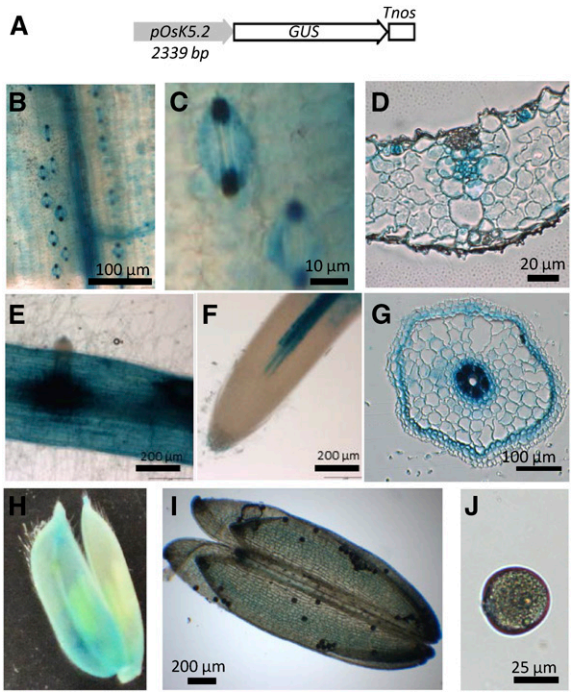

Figure 2. Expression pattern of *OsK5.2* outward Shaker channel gene in rice. A, *Promoter::GUS* construct (promoter region size: 2,339 bp). B to J, GUS activity in transgenic rice plants, revealed by an overnight incubation in the presence of X-Gluc: leaf tissues (B), stomata (C), leaf cross section (D), root (E), root tip (F), root cross section (G), flower (H), anther (I), and pollen (J).

Fig. S1A) 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 wild-type controls. The offspring of the selected plants (*osk5.2* mutants and wild-type controls) were used for in planta experiments. RT-PCR experiments on total RNA prepared from the *osk5.2* mutants and the wild-type control plants confirmed the loss of function of *OsK5.2* in the selected *osk5.2* homozygous mutant lines (Supplemental Fig. S1B).

Impaired K^+ Transport into the Xylem Sap and K^+ Accumulation in Shoots in *osk5.2* Mutant Plants

K^+ was assayed in roots and shoots of 5-week-old plants hydroponically grown on standard 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^+ accumulation in roots but resulted in significantly reduced K^+ contents (by ~25%) in shoots (Fig. 3, A and B, left). The K^+ concentration measurements in xylem sap samples that were collected after leaf excision revealed lower values, by 30% to 40%, in the mutant plants (Fig. 3, A and B, right). Thus, the whole set of data indicated that the absence of *OsK5.2* functional expression resulted in a decrease in K^+ translocation from roots to shoots via the xylem sap.

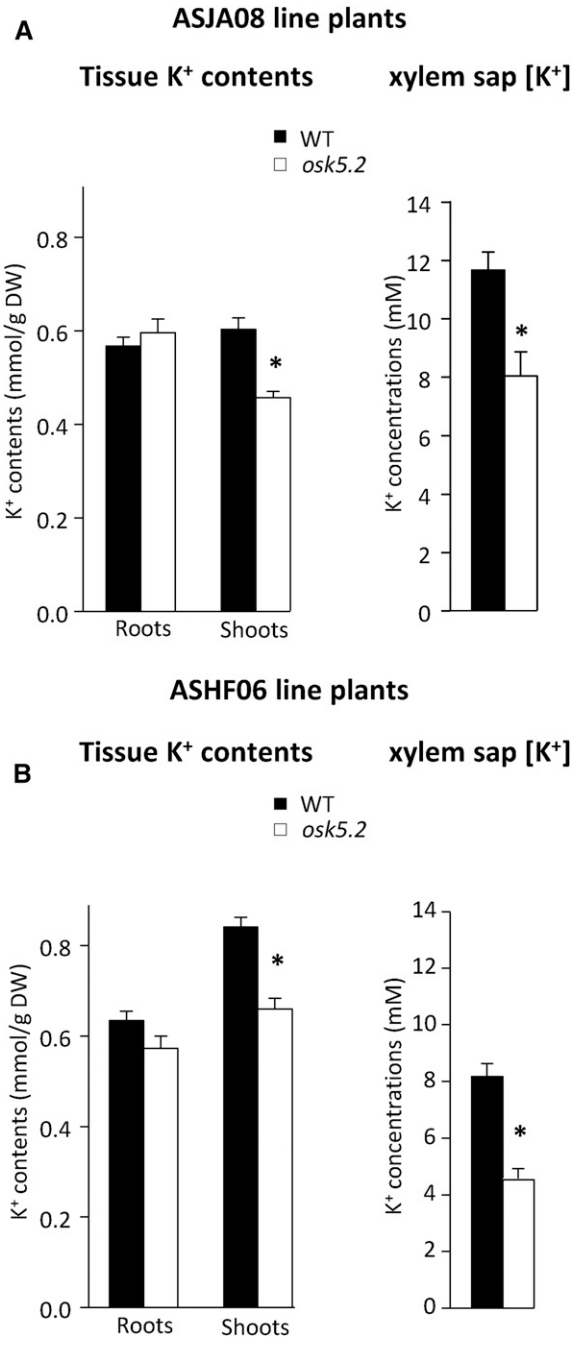

Figure 3. Tissue K^+ contents and xylem sap K^+ concentration in wild-type and *osk5.2* mutant plants. Five-week-old plants were grown in standard Yoshida hydroponic solution. A and B, Root and shoot K^+ contents (left) and xylem sap K^+ concentration (right) in wild-type control (black bars) and *osk5.2* mutant (white bars) plants issued from the initial ASJA08 (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 ASJA08 line, and 2-h exudation in the case of the plants issued from the ASHF06 line. Data are means ± 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^+ content or concentration between the wild-type control and *osk5.2* mutant plants were statistically significant (Student's *t* test, $P < 0.05$).

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 multipotometer (Supplemental Fig. 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).

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

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), and no following transient decrease in transpiration rate was observed (Fig. 6).

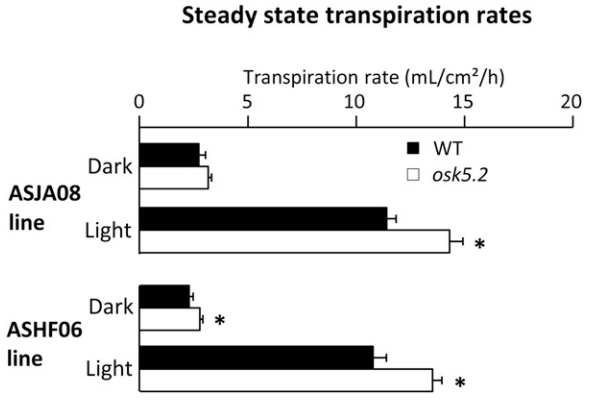

Figure 4. Steady-state transpiration rates in wild-type control and *osk5.2* mutant plants. Five-week-old plants issued from the initial ASJA08 line (above) or ASHF06 line (below) were used for steady-state transpiration rate analyses in dark (~5 h after light was switched off) and in light (~3 h after light was switched on) conditions. The steady-state transpiration rate (in mL/cm^2/h) was determined by dividing the average speed of water loss at steady state (mean of three 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 ± 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 ASHF06 line. An asterisk indicates that the corresponding difference between the wild-type control and mutant plants was statistically significant (Student's t test, $P < 0.05$).

Absence of Shaker-Type Outwardly Rectifying K^+ Channel Activity in Guard Cells of *osk5.2* Mutant Plants

The consequences of *OsK5.2* loss-of-function mutation on the K^+ conductance of the guard cell membrane were analyzed in planta. Guard cells from 2-week-old plants were impaled with double-barreled electrodes (Blatt, 1990; Thiel et al., 1992; Roelfsema et al., 2001), and inward 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, and Fig. 7C), clearly reminiscent of those mediated by the outwardly rectifying K^+ channels from the Shaker family (Gaymard et al., 1998; Hosy et al., 2003; Hedrich, 2012; Véry et al., 2014). Slowly activating outward currents were lacking in *osk5.2* mutant plants (Fig. 7, D and E). In contrast, the inward conductance appeared to be very similar in *osk5.2* mutant and wild-type guard cells (Fig. 7, B and D, right). 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 conditions and thus that *OsK5.2* encodes slowly activating, outwardly rectifying K^+ channels in rice guard cells. It was also observed that the absence of OsK5.2 channel activity caused a positive shift of the free-running membrane potential (cross point with x axis in the current-voltage relationship), by about 50 mV (Fig. 7, C and E).

DISCUSSION

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).

In Arabidopsis, *SKOR* has been reported to be essentially expressed in the root vasculature (Gaymard et al., 1998), and GORK in root periphery cells (except in the root apex) and in stomata (Ache et al., 2000; Ivashikina et al., 2001). The expression pattern of *OsK5.1* in rice is reminiscent of that of *SKOR* in Arabidopsis, with strong expression in root vasculature (Kim et al., 2015). In contrast, *OsK5.2* displays high expression levels in both the root vasculature

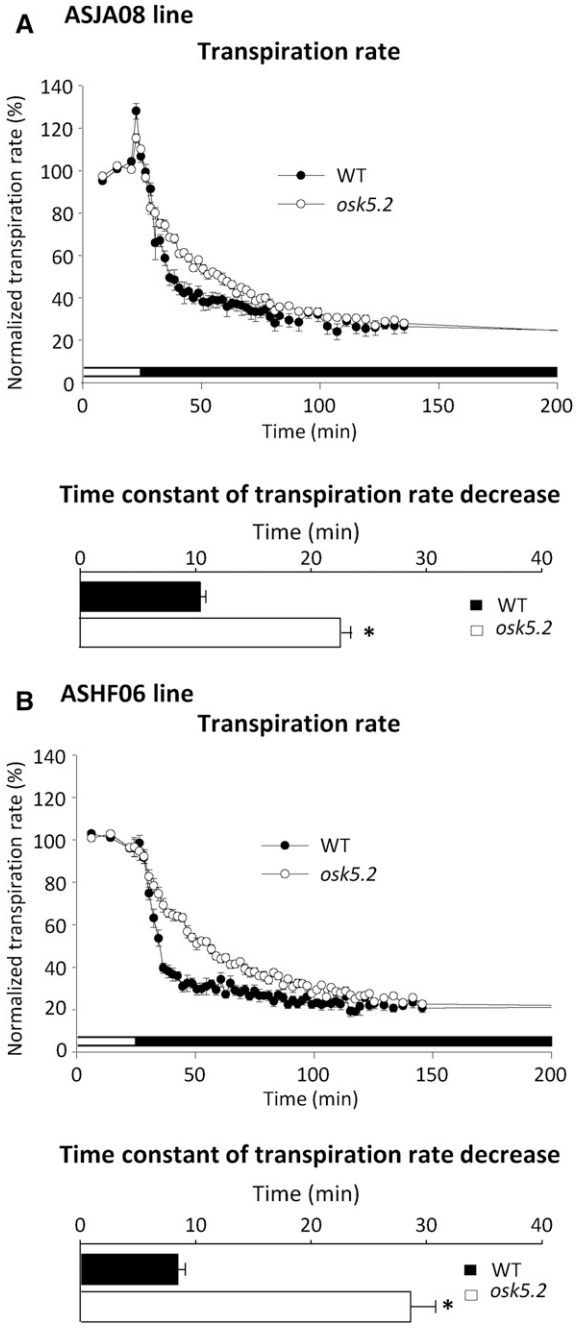

Figure 5. Kinetics of the decrease in transpiration rate upon dark-induced stomatal closure in wild-type and *osk5.2* mutant plants. A and B, Kinetics (top) and time constant (bottom) of the dark-induced decrease in transpiration rate in wild-type control (black circle, black square) and *osk5.2* mutant (white circle, white 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 ± 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, $P < 0.05$).

(Fig. 2, E–G) and the stomata (Fig. 2, B–D); thus, its expression pattern clearly encompasses the patterns reported for both *SKOR* and *GORK* in Arabidopsis (Gaymard et al., 1998; Ache et al., 2000; Ivashikina et al., 2001). It is not yet known whether the expression patterns of *OsK5.1* and *OsK5.2* are shared by genes belonging to the same outward Shaker subgroups from other monocots. The expression pattern of only one maize gene from the *OsK5.2* subgroup has been partially characterized so far, by qRT-PCR analyses (Büchsenschütz et al., 2005). Like *OsK5.2*, this maize outward Shaker gene is expressed in both stomata and roots, and thus its expression pattern may be similar to that of *OsK5.2*.

Functional Properties of OsK5.2 Channels

Members of Shaker group 5 have been cloned in several plant species and successfully expressed in heterologous systems (*Xenopus laevis* oocyte essentially) for electrophysiological analysis of their functional properties (Véry et al., 2014). They all behave as slowly activating, outwardly rectifying, K^+-selective channels. However, so far, all the channels for which such functional analyses have been reported belong to dicotyledonous species. We attempted to express OsK5.2 in *X. laevis* oocytes, but no exogenous current in the oocytes was detected using the classical two-electrode voltage-clamp technique (Véry et al., 1995; Tounsi et al., 2016). This might indicate that these outward K^+ channels have to be coexpressed 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, 2013) and monocots (Boscari et al., 2009; Geiger et al., 2009).

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

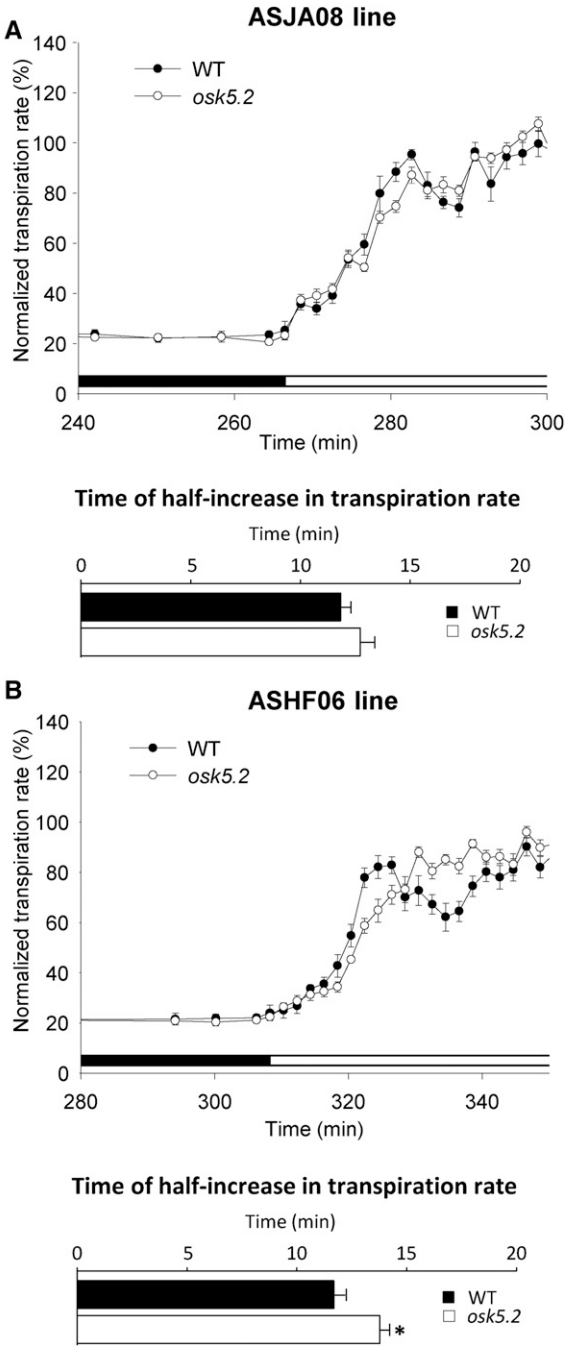

Figure 6. Kinetics of the increase in transpiration rate upon light-induced stomatal opening after a long period of darkness in wild-type and *osk5.2* mutant plants. A and B, Kinetics of light-induced increase in transpiration rate (top) and time of the corresponding half-increase in transpiration rate (bottom) in wild-type control (black circle, black square) and *osk5.2* mutant (white circle, white 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 ± 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, $P < 0.05$).

Comparison of the Roles of OsK5.2 and SKOR in K^+ Translocation toward the Shoots

Both ASJA08 and ASHF06 *osk5.2* loss-of-function mutations caused reduced leaf K^+ 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^+ secretion into the xylem sap in roots, like SKOR in Arabidopsis, and thereby in K^+ translocation toward the shoots. This hypothesis received support from the assay of K^+ 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.

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 to a decrease in the accumulation of K^+ in shoots by about 50% (Gaymard et al., 1998). Thus, the contribution of SKOR to the control of K^+ translocation and accumulation in leaves seems a bit higher than that of OsK5.2 in rice. Since *SKOR* is the only outward Shaker channel gene expressed in the root stele, K^+-permeable channels belonging to other families and/or other types of transport systems (e.g., from the KUP/HAK family; Han et al., 2016) have to significantly contribute to K^+ secretion into the xylem sap in Arabidopsis. The situation may be different in 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^+ translocation toward the shoots.

Comparison of the Roles of OsK5.2 and GORK in Control of Stomatal Aperture and Transpirational Water Loss

Altogether, these 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 Arabidopsis (Hosy et al., 2003). The kinetics of dark-induced decrease in transpiration rate indicated that stomata can close in absence of this outwardly rectifying Shaker-type channel 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^+ efflux systems, but stomatal closure is then less

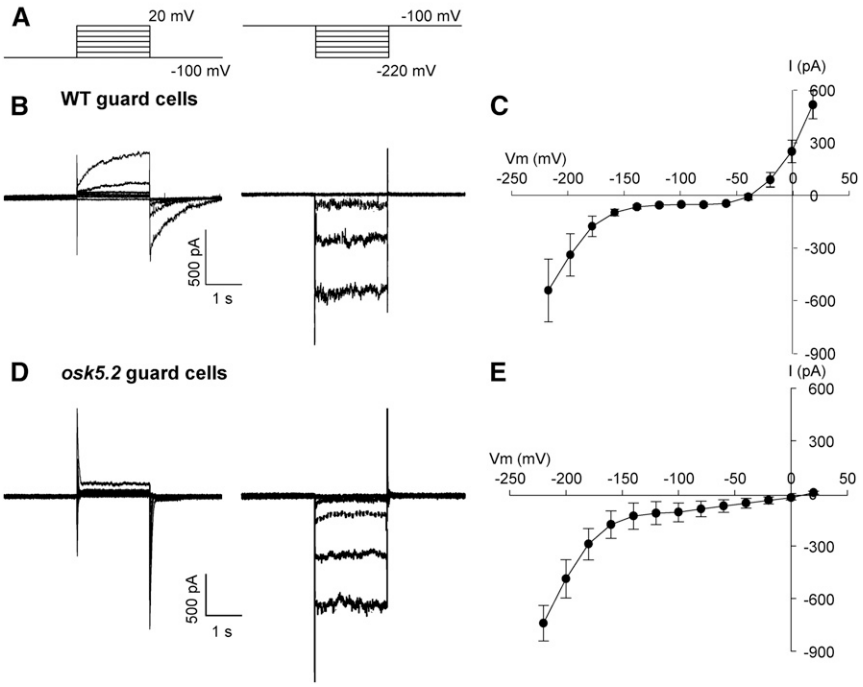

Figure 7. Voltage-clamp experiments with guard cells in intact rice plants. A, Voltage-clamp protocols used to record either outward (left) or inward (right) currents in rice guard cells. Starting at a holding value of −100 mV, the voltage was shifted in 20-mV steps to either +20 mV, or −220 mV. B and 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 ± SE (n = 6 in C, and n = 4 in E).

efficient. The defect in stomatal closure in *osk5.2* mutant plants is also likely to result from the large difference in free running membrane potential, by ~50 mV, between *osk5.2* and wild-type guard cells (Fig. 7). Indeed, in *osk5.2* guard cells, the free-running voltage across the plasma membrane was shifted toward the reversal potential of anion channels, which was approximately +25 mV (as in Kollist et al., 2014). Thus, the driving force for anion efflux (difference between the free running membrane potential and reversal potential of anion channels) was reduced in *osk*5.2 guard cells. This is expected to cause a decrease in anion efflux, and thus in the rate of stomatal closure.

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^+ channel activity in rice is also likely to result in larger steady-state stomatal aperture in light, based on the observation that the steady-state transpiration rate was increased (Fig. 4). This would mean that OsK5.2 outwardly rectifying 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 decrease in turgor of the leaf cells. A decrease in the turgor of epidermal and subsidiary cells more important than that of guard cells could result in reduced back-pressure on stomatal guard cells and, thus, in increased steady-state stomatal aperture in the light. The impact of OsK5.2 activity on steady-state transpiration rate in light could be understood within the framework of this latter hypothesis.

CONCLUSION

By controlling the transpirational water loss at leaf surface, stomata also regulate the flux of water from roots to shoots within the xylem vasculature, and thereby contribute to the regulation of nutrient ion translocation toward the shoots. In Arabidopsis, various ion transport systems play a role in both ion translocation into the xylem sap toward the shoots and ion transport across the guard cell membrane and control of stomatal aperture. This is the case, for example, of the anion channel SLAH3 (Geiger et al., 2011; Cubero-Font et al., 2016), the nitrate transporter 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^+, some kind of specialization has occurred within the Shaker group 5 in Arabidopsis since K^+ release from guard cells for stomatal closure has been strictly assigned to GORK, and K^+ secretion into the xylem sap to SKOR. In rice, OsK5.2 contributes to both K^+ release by guard cells for stomatal closure and K^+ translocation toward the shoots. The physiological significance of such evolutionary differences would be interesting to clarify. The organization and the roles of the Shaker family are strongly conserved in plants, allowing one to assume functional equivalence between orthologs, but these data clearly indicate that the biological variability deserves to be investigated, since differences between plant species in the use of this gene family are likely to play a role in major physiological functions and in adaptation to environmental constraints.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Rice Nipponbare cultivar (*Oryza sativa* 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-liter containers [0.7 mM KNO_3, 0.8 mM KH_2PO_4, 1.2 mM $Ca(NO_3)_2$, 0.5 mM $(NH_4)_2SO_4$, 1.6 mM $MgSO_4$, 60 μM $Na_2FeEDTA$, 45 μM H_3BO_3, 20 μM $MnSO_4$, 1.6 μM $CuSO_4$, 1.4 μM $ZnSO_4$, and 0.3 μM $(NH_4)_6Mo_7O_{24}$, 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 μmol m^{-2} s^{-1}, 80% relative humidity).

Construction of Reporter Gene Constructs and Transgenic Plants

OsK5.2 promoter region (2,339 bp) was amplified by PCR from rice Nipponbare genomic DNA using the primers listed in Supplemental Table S1. The PCR product was then cloned into pGemTeasy vector for sequencing before subcloning into destination vector. The promoter was then transferred upstream the *GUS* reporter gene in pCAMBIA1301 vector, where it replaced CAMV35S promoter. The resulting plasmid was introduced into *Agrobacterium tumefaciens* EHA105 strain for rice transformation, as described by Sallaud et al. (2003). Briefly, dehulked rice seeds were rinsed in ethanol 70% for 90 s and treated with disinfection solution (50 g/L of sodium hypochlorite) for 30 min. After sterilization, the seeds were transferred to a callus induction medium, leading to callus development from the scutellum region. Rice grains with developing calluses were incubated with bacteria suspension for 90 to 180 s and cocultured during 3 d. After the coculture period, developing calluses were washed with carbenicillin (500 mg/L), separated, divided into small pieces, and transferred to shoot induction medium containing hygromycin for transgenic tissue selection. Shoots regenerated from calluses were separated and transferred to rooting medium. Four-week-old plantlets were transferred to soil and grown in greenhouse for multiplication and selection of homozygous plants in the next generation using antibiotics leaf painting method (Cotsaftis et al., 2002).

Histochemical Analysis of GUS Activity

Leaf samples of 4-week-old plants and flowers were collected from homozygous plants grown in a greenhouse. Root samples were collected from 1-week-old plants grown in standard Yoshida hydroponic medium. All samples were submerged in GUS solution (50 mM phosphate buffer, pH 7, 2.5 mM ferricyanide, 2.5 mM ferrocyanide, 0.05% [v/v] Triton X-100, and 1 mM X-Gluc) contained in a 24-well plate, the leaves having been previously quickly divided into 1 × 1-cm pieces. The plate was placed under vacuum for 30 min to facilitate the penetration of GUS solution into the tissues, and then the samples were incubated overnight at 37°C. After the incubation 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), from GUS-stained fixed samples (prefixation in 50 mM phosphate buffer, pH 7, 1.5% formaldehyde, and 0.05% Triton X-100; fixation in 75 mM phosphate buffer, 2% paraformaldehyde, and 0.5% glutaraldehyde) embedded in Technovit 7100 resin (Kulzer).

Selection of KO Mutant and Wild-Type Control Plants

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

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 2 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 mounted onto the table of an upright microscope (Axoiskop 2FS; Zeiss). About 0.5 to 1 mL of bath solution (5 mM potassium citrate, 5 mM KCl, and 0.1 mM $CaCl_2$, pH 5.0) was placed between the leaf and the water immersion objective (Achroplan 40/0.80W; Zeiss), used to visualize the stomata. Stomatal opening was induced by light provided by the microscope lamp. Guard cells were impaled using double-barreled electrodes prepared from 1-mm-diameter glass capillaries (1403547; Hilgenberg) filled with 300 mM KCl. A glass capillary filled with 300 mM KCl and plugged with 300 mM KCl in 2% agarose was placed in the drop of solution between the leaf and the objective and served as a reference electrode. The series resistance present between leaf apoplast and reference electrode was estimated by measuring the resistance between two drops of bath solution placed on the leaf surface, at a distance of ~1 cm. This approach revealed a series resistance (which was not different between wild type and *osk5.2* mutant plants) of 1.3 ± 0.1 (n = 5) MΩ at the abaxial leaf face. With guard cell currents <1 nA (Fig. 7), such a series resistance leads to an overestimation of the applied voltage of <2 mV. The potential difference between the bath solution and guard cell apoplast ("surface" potential) was −10 ± 2 mV (n = 6). Because the series resistance and potential difference between the external solution and the apoplast appeared as low, no attempt was made to correct their impact on current-voltage plots. The Win WCP V5.1.6 software (J. Dempster, Strathclyde University, Glasgow, UK) was used to set up the protocol and record applied voltage and current traces, using an ITC-16 interface (Instrutech) and a GeneClamp 500 (Axon Instruments) amplifier.

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 5 weeks. One day before the experiment, plants were transferred into a multipotometer device (Supplemental Fig. 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 min of recording and was maintained for 4 h before switching back to light condition.

Analysis of K^+ Content in Tissues and Xylem Sap

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

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

Accession Numbers

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

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Selection of knockout mutant plants for *OsK5.2* gene.

Supplemental Figure S2. The multipotometer 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

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