A Magnesium Transporter OsMGT1 Plays a Critical Role in Salt Tolerance in Rice1[OPEN]

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Salt stress is one of the major factors limiting rice (Oryza sativa) production globally. Although several transporters involved in salt tolerance have been identified in rice, the mechanisms regulating their transport activity are still poorly understood. Here, we show evidence that a rice Mg transporter OsMGT1 is required for salt tolerance probably by regulating transport activity of OsHKT1;5, a key transporter for the removal of Na+ from the xylem sap at the root mature zone. Knockout of OsMGT1 did not affect total Na uptake, but increased Na concentration in the shoots and xylem sap, resulting in a significant increase in salt sensitivity at low external Mg2+ concentration (20–200 μM). However, such differences were abolished at a higher Mg2+ concentration (2 mM), although the total Na uptake was not altered. OsMGT1 was expressed in both the roots and shoots, but only that in the roots was moderately up-regulated by salt stress. Spatial expression analysis revealed that OsMGT1 was expressed in all root cells of the root tips but was highly expressed in the pericycle of root mature zone. OsMGT1 was also expressed in the phloem region of basal node, leaf blade, and sheath. When expressed in Xenopus laevis oocytes, the transport activity of OsHKT1;5 was enhanced by elevating external Mg2+ concentration. Furthermore, knockout of OsHKT1;5 in osmt1 mutant background did not further increase its salt sensitivity. Taken together, our results suggest that Mg2+ transported by OsMGT1 in the root mature zone is required for enhancing OsHKT1;5 activity, thereby restricting Na accumulation to the shoots.

Soil salinity is a major abiotic stress limiting the yield of agricultural crops worldwide. Approximately 7% of the world’s total land area is affected by high salt stress (Shabala and Cuin, 2008; Munns and Tester, 2008). High salt concentration inhibits plant growth through osmotic stress and ionic Na+ stress (Munns and Tester, 2008; Horie et al., 2012). Osmotic stress causes inhibitions of water uptake, cell elongation, and leaf development, while ionic stress results in high Na accumulation in the shoots, which decreases protein synthesis, enzymatic reactions, and photosynthetic processes (Zhu, 2001; Horie et al., 2012; Deinlein et al., 2014). Therefore, restricting Na accumulation to the shoots (especially in the leaves) is important to protect plants from ionic Na+ stress (Davenport et al., 2005; Yamaguchi et al., 2013).

Rice (Oryza sativa) is the most salt-sensitive species among cereals (Munns and Tester, 2008; Lutts et al., 1996), but several genes involved in salt tolerance in rice have been identified. OsSOS1 in rice was isolated based on the homology with AtSOS1 in Arabidopsis (Arabidopsis thaliana; Martínez-Atienza et al., 2007; Shi et al., 2000). Similar to AtSOS1, OsSOS1 also encodes a plasma membrane-localized Na+/H+ antiporter, which mediates active Na+ extrusion in the roots under salt stress (Shi et al., 2000). Although the phenotype of OsSOS1 knockout lines under salt stress is unknown, OsSOS1 was able to complement salt tolerance of atsos1 mutant, indicating that OsSOS1 also plays an important role in salt tolerance in rice (Martínez-Atienza et al., 2007). Furthermore, the CBL-interacting protein kinases OsCIPK24 and the calcineurin B-like protein OsCBL4, which are homologs of AtSOS2 and AtSOS3 in Arabidopsis, respectively, act coordinately to activate OsSOS1 transport activity. This SOS-mediated pathway may

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represent a basic salt tolerance in both dicots and monocots. After Na\(^+\) uptake, a fraction of Na\(^+\) taken up is sequestered into the vacuoles of both root and shoot cells by AtNHX1, a vacuolar Na\(^+\), K\(^+/\)H\(^+\) exchanger in Arabidopsis (Apse et al., 1999, 2003; Leidi et al., 2010). Its homolog in rice has also been implicated in salt tolerance, although the exact role is not well understood (Chen et al., 2007).

Several members of HKT gene subfamily in rice, including OsHKT1;1, OsHKT1;3, OsHKT1;4, and OsHKT1;5 have also been implicated in salt tolerance in rice (Hamamoto et al., 2015). All these members function as a Na\(^+\) influx transporter but have different expression patterns. OsHKT1;1 encodes a plasma membrane-localized protein and was mainly expressed in the plasmodium of leaf blades (Wang et al., 2015). Knock-out of this gene resulted in increased salt sensitivity and Na accumulation in the shoots, indicating that OsHKT1;1 is involved in retrieving Na\(^+\) from the leaf blade (Wang et al., 2015). Furthermore, its expression was regulated by an MYB-type transcription factor (OsMYBc). By contrast, OsHKT1;3 was localized to the Golgi (Rosas-Santiago et al., 2015). It is expressed in the vascular tissues of roots and leaves (Jabnoune et al., 2009), but its exact role in salt tolerance is unknown. OsHKT1;4 is mainly expressed in the leaf sheath and encodes a plasma membrane-localized protein (Suzuki et al., 2016). Recent functional analysis showed that OsHKT1;4 does not contribute to salt tolerance at the vegetative growth stage (Suzuki et al., 2016); however, at the reproductive stage, knockdown of OsHKT1;4 resulted in increased Na accumulation in the leaf sheath and leaf blade under salt stress, implicating that this gene may be involved in the Na\(^+\) exclusion in the leaf sheath (Suzuki et al., 2016). On the other hand, SKC1/OsHKT1;5 was suggested to be a quantitative trait locus controlling a higher K\(^+\)/Na\(^+\) ratio in the shoots (Ren et al., 2005). In contrast to OsHKT1;4, SKC1/OsHKT1;5 is highly expressed in the roots (Ren et al., 2005). Furthermore, it is preferentially expressed in the parenchyma cells surrounding the xylem vessels. Similar to AtHKT1;1 in Arabidopsis (Mäser et al., 2002; Sunarpi et al., 2005) and TmHKT1;5-A and TaHKT1;5-D in wheat (Triticum aestivum; James et al., 2006, 2011; Byrt et al., 2014), SKC1/OsHKT1;5 is therefore thought to be responsible for retrieving Na\(^+\) from the xylem sap, leading to low Na accumulation in the shoots (Ren et al., 2005; Deinlein et al., 2014). All these studies show that these HKT genes play important roles in salt tolerance in different organs and tissues of rice; however, the mechanisms regulating the transport activity of HKT proteins are still poorly understood.

MGT family proteins have been known as Mg transporters in both prokaryote and eukaryote (Hmiel et al., 1986; Li et al., 2001). There are 10 MGT homologs in the Arabidopsis genome and nine homologs in rice (Schock et al., 2000; Gebert et al., 2009). Among them, AtMGT6 in Arabidopsis and OsMGT1 in rice mediate root Mg uptake, respectively, although they differ in their gene expression patterns (Mao et al., 2014; Chen et al., 2012). In this study, we found that OsMGT1 is involved in enhancing OsHKT1;5 transport activity at least in rice. OsMGT1 is a plasma membrane-localized transporter for Mg\(^{2+}\) (Chen et al., 2012; Chen and Ma, 2013). It was initially found to be involved in Al tolerance. Rapid up-regulation of OsMGT1 expression in response to Al stress resulted in increased cytosolic Mg concentration, thereby preventing Al binding to enzymes and other cellular components (Chen et al., 2012; Chen and Ma, 2013). Further detailed analysis of this gene revealed that OsMGT1 is also required for conferring salt tolerance in rice through enhancing the transport activity of OsHKT1;5, a key player of salt tolerance in rice.

RESULTS

Knockout of OsMGT1 Resulted in Hypersensitivity to Salt Stress

To investigate the role of OsMGT1 in salt tolerance in rice, we grew both the wild-type rice and two OsMGT1 knockout lines in a nutrient solution containing 0.27 mm Mg\(^{2+}\) in the presence or absence of 50 mm NaCl. In the absence of NaCl, the growth was similar between the two independent OsMGT1 knockout lines (osmgt1) and wild type (Fig. 1A). However, in the presence of 50 mm NaCl, the old leaves of osmgt1 mutants were wilted after exposure to 12 d (Fig. 1A and B), but that of wild type was hardly affected. The dry weight of the roots and shoots was 34% and 22% lower in the mutants than in the wild type (Fig. 1C).

To examine the possibility that the decreased growth in the mutants is caused by osmotic stress, we grew them in a nutrient solution containing high concentrations of mannitol. Although the growth was inhibited by high concentration of mannitol, there was no difference in the growth between the wild type and mutants (Supplemental Fig. S1A). There was also no difference in the water loss rate of detached leaves (Supplemental Fig. S1B). These results indicate that decreased salt tolerance in osmgt1 mutants is caused by Na\(^+\) toxicity rather than osmotic stress. In addition, we also investigated the sensitivity of osmgt1 mutants to high Mg\(^{2+}\) (10 mM) and Cd\(^{2+}\) (5 μM) toxicity (Supplemental Fig. S2). High Mg and Cd inhibited the growth of both wild type and osmgt1 mutants, but there was no difference in the tolerance to them between wild type and mutants, indicating that OsMGT1 plays a specific function in Na\(^+\) tolerance.

osmgt1 Mutant Showed Higher Na Accumulation in the Shoots

The total Na uptake (the total Na content per plant) was similar between the mutants and wild type (Fig. 2A). However, Na concentration of the shoots was 1.5-fold higher in the mutants than in the wild type, although that of the roots was similar (Fig. 2B). As a result, the root-to-shoot translocation of Na (the percentage of shoot Na content in total Na content in plant)
was 96% in the mutants, in contrast to 93% in the wild type (Fig. 2C). The Na concentration in xylem sap was also higher in the mutants than in the wild type (Fig. 2D). By contrast, Mg concentration in mutant roots was lower than wild type, although that in the shoots was similar (Supplemental Fig. S3A). K concentration in the roots and shoots was similar between the mutant and wild type (Supplemental Fig. S3B). There was also no difference in Mg and K concentration in the xylem sap between wild type and mutants (Supplemental Fig. S3, C and D). Furthermore, no difference was found in the concentration of other mineral elements in both the roots and shoots between wild type and mutants (Fig. 2E).

Leaf age-dependent analysis showed that Na accumulation in the leaf blade increased with the age in both the wild type and mutants (Fig. 3A), but the mutants always accumulated higher Na than the wild type in each leaf. Furthermore, with the help of the scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDS), a stronger signal of Na was detected in the old leaves of the mutants than those of the wild type (Fig. 3B).

High Mg²⁺ Restored the Salt Tolerance of osmgt1 Mutants

To investigate whether salt toxicity in the knockout lines can be alleviated by Mg²⁺, we exposed the seedlings to a solution containing low (0.02, 0.2 mM) and high (2 mM) Mg²⁺ in the presence of 50 mM Na⁺. At low external Mg²⁺ concentrations, the growth of osmgt1 mutants was significantly inhibited compared with wild type (Fig. 4A). However, at high Mg²⁺ condition, the growth of osmgt1 mutant was restored to the level similar to the wild type (Fig. 4A). The total Na uptake was not altered by increasing external Mg²⁺ concentrations (Fig. 4B), and there was also no difference between the wild type and mutants. The Na concentration in the shoots of osmgt1 mutants was higher than that in the wild type at low Mg²⁺ concentrations but was decreased by nearly 50% at high Mg²⁺ concentration (Fig. 4C) and became similar to that of wild type at 2 mM Mg. By contrast, the Na concentration in the shoots of wild type was not affected by external Mg²⁺ concentrations (Fig. 4C). The Na concentration in the roots was similar between wild type and mutants at all Mg²⁺ concentrations and was not affected by Mg²⁺ concentrations (Fig. 4D). We also compared Mg concentration of wild type and osmgt1 mutants. The Mg concentration in the roots increased with increasing Mg²⁺ supply in all lines, although osmgt1 mutants always showed a significantly lower Mg concentration than wild type at each Mg²⁺ supply (Fig. 4E). However, at 2 mM Mg, Mg concentration in the roots of osmgt1 mutants reached to a similar level of the wild type at 0.2 mM Mg (Fig. 4E). By contrast, there was no difference in the shoot Mg concentration between wild type and mutants, although it was also increased with increasing external Mg²⁺ concentrations (Fig. 4F).
OsMGT1 Expression Was Moderately Up-Regulated in Response to Salt Stress

OsMGT1 was expressed in both the shoots and roots, but only that in the roots was up-regulated by about 2-fold after exposure to 50 mM NaCl for 24 h (Fig. 5A). A dose-response experiment showed that OsMGT1 expression was not induced by low external NaCl concentrations (<10 mM) but induced by high NaCl concentrations (>30 mM; Fig. 5B). A time-course experiment showed that the induction of OsMGT1 occurred at 3 h after the exposure to 50 mM NaCl and the expression was increased with exposure times (Fig. 5C).

Spatial expression analysis revealed that the expression of OsMGT1 was higher in the root mature zones than in the root tips, but the expression in all root regions was up-regulated by NaCl (Fig. 5D). We also compared root spatial expression pattern of some salt tolerance genes. OsHKT1;5 was highly expressed in the root mature zone and its expression was up-regulated by NaCl exposure (Fig. 5E). By contrast, OsHKT1;4 was down-regulated by NaCl (Fig. 5F), and the expression of OsSOS1 was not affected by NaCl exposure (Fig. 5G). The expression of both OsHKT1;4 and OsSOS1 was also higher in the mature root region than the root tips (Fig. 5, F and G). In the osmgt1 mutant, the expression of OsHKT1;4, OsHKT1;5, and OsSOS1 was not altered compared to the wild type under both salt and nonsalt stress (Supplemental Fig. S4).

Tissue Specificity of Expression of OsMGT1

To examine the tissue and cell specificity of OsMGT1 expression, we generated transgenic rice lines carrying the 2.5 kb promoter sequence of OsMGT1 fused with GFP. Immunostaining of the transgenic rice with a GFP antibody showed that the signal was detected in the whole root, but with different patterns (Fig. 6). In the root tips, the signal was observed in all root cells (Fig. 6,
indicated that a change of Mg\(^{2+}\) concentration in and 2 at the membrane potentials of currents were found in in the 2 mM Na\(^+\) bath solution elicited larger inward increase of the Mg\(^{2+}\) concentration from 1 mM to 10 mM concentrations tested (Fig. 7, A and B). In contrast, an transport in Mg\(^{2+}\) Enhances OsHKT1;5-Mediated Na\(^+\) Transport

OsHKT1;5 is a key player for salt tolerance in rice (Ren et al., 2005). To link OsHKT1;5 and OsMGT1, we tested the effect of Mg\(^{2+}\) on OsHKT1;5-mediated Na\(^+\) transport in X. laevis oocytes by two-electrode voltage clamp (TEVC; Fig. 7). Water-injected control oocytes showed small background currents in the presence of 1 mM and 10 mM Mg\(^{2+}\) with 2 mM Na\(^+\), and no characteristic difference was found between the two Mg\(^{2+}\) concentrations tested (Fig. 7, A and B). In contrast, an increase of the Mg\(^{2+}\) concentration from 1 mM to 10 mM in the 2 mM Na\(^+\) bath solution elicited larger inward and outward currents from OsHKT1;5-expressing oocytes (Fig. 7, C and D). Significant increases in inward Na\(^+\) currents were found in OsHKT1;5-expressing oocytes at the membrane potentials of \(-105, -120, -135,\) and \(-150\) mV upon the 10-fold Mg\(^{2+}\) increase in the bath (\(P < 0.05;\) Fig. 7E, inset). Current voltage relationships indicated that a change of Mg\(^{2+}\) concentration in the bath solution leads to no remarkable influence on the reversal potential of water-injected oocytes and OsHKT1;5-expressing oocytes (Fig. 7E). In addition, increasing Mg\(^{2+}\) concentration from 0.1 to 1 mM did not significantly increase inward and outward currents from OsHKT1;5-expressing oocytes (Supplemental Fig. S5). These results suggest that high Mg\(^{2+}\) concentration at millimolar level is required to enhance the OsHKT1;5-mediated Na\(^+\) transport.

Mg\(^{2+}\) Enhances OsMGT1-Mediated Na\(^{+}\) Transport

To further confirm the relationship between OsHKT1;5 and OsMGT1, we compared the phenotype of single knockout mutant of OsHKT1;5 and OsMGT1, and a double mutant of OsHKT1;5 and OsMGT1 under salt stress. There was no difference in the growth among wild type, single, and double mutants in the absence of NaCl (Fig. 8, A and B). However, in the presence of NaCl, the lower leaves of single and double mutants were rolled and wilt. The dry weight was decreased by about 41% by NaCl in the single and double mutants in contrast to 21% decrease in wild type (Fig. 8B). The Na concentration in the shoots of single and double mutants was about 2-fold of wild type (Fig. 8C). There were no differences in the dry weight and Na accumulation in the shoots between single and double mutants (Fig. 8, B and C). We further investigated whether high Mg\(^{2+}\) can alleviate salt stress-induced growth inhibition in oshkt1;5 and the double mutant (Supplemental Fig. S7). Different from osmg1 mutants, high Mg supply did not alter the salt sensitivity in oshkt1;5 and the double mutant (Supplemental Fig. S7), suggesting that Mg\(^{2+}\) restoration in rice salt tolerance is specific to OsMGT1 function.

OsHKT1;5 Knockout Line Shows Similar Phenotype to OsMGT1 Knockout Line under Salt Stress

A and B), whereas in the root mature zone, strong signal was only detected in the stellar tissues, especially in the pericycle (Fig. 6, C and D). No signal was detected in the roots of wild-type rice (Fig. 6E), indicating high specificity of this antibody. The signal in the root mature zone was enhanced by salt stress (Fig. 6, F and G). In addition, the signal was also observed in the phloem region of basal node (Fig. 6H), leaf sheath (Fig. 6, I and J), and leaf blade (Fig. 6K).

A representative image of six shoot samples is presented. Bar = 500 μm.
DISCUSSION

Different Roles of OsMGT1 in Al Tolerance and Salt Tolerance

OsMGT1 is a plasma membrane-localized Mg\textsuperscript{2+} transporter and was found to be involved in Al tolerance in rice previously (Chen et al., 2012). Knockout of OsMGT1 resulted in decreased Mg in the roots and increased sensitivity to Al, although the shoot Mg concentration was not altered. In this study, we found that OsMGT1 was also involved in salt tolerance. In two independent knockout lines of OsMGT1, the sensitivity to salt stress was significantly increased (Fig. 1A). Especially, the lower leaves of the mutants were wilted due to Na\textsuperscript{+} toxicity. However, it seems that OsMGT1 plays different roles in Al tolerance and salt tolerance.

Figure 4. Alleviation of salt stress by high Mg\textsuperscript{2+} supply in OsMGT1 knockout lines. A and B, Dry weight (A) and total Na content (B) per plant. C and D, Na concentration in shoots (C) and roots (D). E and F, Mg concentration in roots (E) and shoots (F). Seedlings of both wild-type rice (WT) and two OsMGT1 knockout lines (osmgt1-1 and osmgt1-2) were exposed to a nutrient solution containing 0.02, 0.2, or 2 mM MgCl\textsubscript{2} in the presence of 50 mM NaCl for 7 d. Data are means ± sd (n = 3). Means with different letters are significantly different (P < 0.05 by Tukey’s test).
The site of $\text{Al}^{3+}$ toxicity, which is characterized by rapid root elongation inhibition, is located at the root tips (Ryan et al., 1993). Therefore, OsMGT1 expressed at the root tip is responsible for Al tolerance, which is supported by the expression pattern of OsMGT1. OsMGT1 is up-regulated by $\text{Al}^{3+}$ in the root tips (Chen et al., 2012), and its expression is regulated by ART1, a C2H2 zinc-finger type transcription factor (Yamaji et al., 2009). Furthermore, it is localized to the all cells of the root tips (Fig. 6, A and B). This localization is similar to other ART1-regulated genes (Huang et al., 2009, 2012; Xia et al., 2010, 2013; Yokosho et al., 2011; Che et al., 2016). Therefore, $\text{Al}^{3+}$-induced up-regulation of OsMGT1 in the root tips results in increased cytosolic $\text{Mg}^{2+}$

**Figure 5.** Gene expression pattern of OsMGT1 in response to salt stress. A, Expression of OsMGT1 in the roots and shoots. Rice seedlings were exposed to a solution containing 50 mM NaCl for 24 h. B, Dose-response expression of OsMGT1 in rice roots. Rice seedlings were exposed to a solution containing different NaCl concentrations for 6 h. C, Time-dependent expression of OsMGT1 in rice roots. Rice seedlings were exposed to a solution containing 50 mM NaCl for different time. D to G, Root spatial expression pattern of OsMGT1 (D), OsHKT1;5 (E), OsHKT1;4 (F), and OsSOS1 (G). Different segments (0–5, 5–10, and 10–20 mm from root tips) were excised after the roots exposed to 0 or 50 mM NaCl for 24 h. The expression level was determined by real-time RT-PCR. Data are means $\pm$ SD ($n = 3$). The asterisk shows a significant difference compared with $-\text{Na}$ in A, B, and D to F ($P < 0.05$ by Tukey’s test).
Figure 6. Tissue-specific and Na-responsive expression of OsMGT1. A to E, Expression of OsMGT1 in different root region. Immunostaining with an anti-GFP antibody was performed in different root zone of pOsMGT1-GFP transgenic rice (A–D) and wild-type rice (E), including longitudinal section of root (A), cross sections at 5 mm (B and E), and 30 mm (C and D) from root apex. F to K, Expression of OsMGT1 expression to salt stress. Immunostaining of root segments (20 mm from root tips; F and G), basal node (H), leaf sheath (I and J), and leaf blade (K), after exposure to 0 (F) or 50 mM (G–K) NaCl for 24 h. Red color shows signal from GFP antibody detected with a secondary antibody. Cyan color shows cell wall autofluorescence. Yellow-dotted areas in C and I were magnified in D and J, respectively. EN, Endodermis; EX, exodermis. Five independent transgenic lines were investigated, and the representative results are shown. Bars = 500 μm (A) and 100 μm (B–K).
concentration, which prevents Al$^{3+}$ binding to oxygen donor compounds because Al$^{3+}$ and Mg$^{2+}$ ions have similar hydrated radius (Bose et al., 2011).

By contrast, OsMGT1 expressed in the root mature region is responsible for salt tolerance. The root mature region has a developed vascular system and is the zone responsible for the root-to-shoot translocation of Na. The expression of OsMGT1 in the root mature zone was up-regulated by salt stress, although the induction was not as large as that by Al$^{3+}$ stress (Fig. 5; Chen et al., 2012). Furthermore, different from the root tips, OsMGT1 in the root mature zone was highly expressed in the stellar tissues, especially in the root pericycle, which connects to the xylem and phloem (Fig. 6, C and D). Knockout of OsMGT1 resulted in significant high root-to-shoot translocation of Na, leading to the accumulation of Na in the leaves (Figs. 2B and 3A). These findings indicate that OsMGT1 in the root mature zone is involved in restriction of Na to the shoots. Therefore, the physiological role of OsMGT1 depends on its spatial expression in the roots.

OsMGT1 Confers Salt Tolerance through OsHKT1;5-Mediated Xylem Na$^+$ Unloading in Roots

Several salt tolerance genes in rice have been reported including OsSOS1, OsHKT1;1, OsHKT1;4, and OsHKT1;5, which function as Na$^+$ transporters (Martinez-Atienza et al., 2007; Wang et al., 2015; Suzuki et al., 2016; Ren et al., 2005). There is a possibility that Mg$^{2+}$ transported by OsMGT1 regulates the transport activity of these transporters. OsSOS1 as a Na$^+_/$H$^+$ antiporter was proposed to be responsible for extruding Na$^+$ from the roots (Martinez-Atienza et al., 2007). The
expression of OsSOS1 in the roots was not induced by NaCl within 24 h, and there was also no difference in the expression level of OsSOS1 between wild type and two osmgt1 mutants (Fig. 5G; Supplemental Fig. S4C). Since there was no difference in the total Na uptake between wild type and two osmgt1 mutants (Fig. 2A), it is unlikely that OsSOS1 activity is associated with OsMGT1. On the other hand, OsHKT1;1 was mainly expressed in the shoots (Wang et al., 2015), and the contribution of OsHKT1;4 to the Na exclusion from shoots was found to be low at the vegetative growth stage (Suzuki et al., 2016). Therefore, the possibility of these transporters in restricting Na accumulation through OsMGT1 is also excluded at the young seedling stage. By contrast, our results showed that the activity of OsHKT1;5 requires cooperation of OsMGT1.

This is supported by several pieces of direct and indirect evidence. First, the activity of OsHKT1;5 was significantly enhanced by Mg2+ in TEVC experiments (Fig. 7). Second, increased salt sensitivity and Na accumulation in the shoots of osmgt1 mutants were completely restored at high Mg2+ supply (Fig. 4). Third, OsMGT1 expression showed similar tissue localization at the root stele as OsHKT1;5 in the root mature zones (Ren et al., 2005; Fig. 6, C and D). Fourth, knockout of OsHKT1;5 or OsMGT1 resulted in increased Na accumulation in the shoots similarly (Fig. 8C). Fifth, knockout of OsHKT1;5 in the background of osmgt1 mutant did not further increase salt tolerance and Na accumulation in the shoots (Fig. 8). OsHKT1;5 has been strongly suggested to play a key function in restricting Na accumulation in the shoots by excluding Na+ from the xylem, and its
differential expression is also responsible for genotypic difference in salt tolerance of rice (Ren et al., 2005; Coutsifis et al., 2012; Deinlein et al., 2014). However, the expression of OsHKT1;5 did not change in osmgt1 mutants (Supplemental Fig. S4B). Our results therefore indicate that Mg$^{2+}$ transported by OsMGT1 is required for enhancing OsHKT1;5 activity in the stellate tissues of root mature zone, conferring salt tolerance in rice. The exact mechanism underlying this activation is unknown, but the possible mode is that Mg$^{2+}$ is required for the stereostructure for OsHKT1;5 protein.

In addition to expression of OsMGT1 in the roots, its expression was also detected in the shoots, although it was not induced by salt stress (Fig. 5A). Furthermore, OsMGT1 was expressed in the phloem region of basal node, leaf blade, and sheath (Fig. 6, H–K). This tissue specificity of expression of OsMGT1 is similar to OsHKT1;1 and OsHKT1;4 (Wang et al., 2015; Suzuki et al., 2016). Therefore, there is a possibility that Mg$^{2+}$ transported by OsMGT1 in these tissues also plays a role in enhancing the transport activity of OsHKT1;1 and OsHKT1;5, although further work remains to be done.

In conclusion, our results showed that OsMGT1 is involved in salt tolerance in rice by enhancing at least OsHKT1;5 activity. Mg$^{2+}$ transported by OsMGT1 in the stellate tissues of the root mature zone enhances the Na$^+$ transport activity of OsHKT1;5, resulting in decreased Na accumulation to the shoots and subsequently contributing to increased salt tolerance in rice.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two Tos-17 insertion lines, NF0595 (osmgt1-1) and NE4528 (osmgt1-2) for OsMGT1, were obtained from the Rice Genome Resource Center in Japan. The homozygous insertion lines were isolated by PCR according to Chen et al. (2012). The osmgt1-1 mutant was a T-DNA insertion line (Supplemental Fig. S8A). RT-PCR was performed using gene-specific primers (RT-F, 5’-TGTAGTACCTGCCACCTTAC-3’; RT-R, 5’-ATGTGAGAACCGCTGTAGT-3’) to genotype osmgt1 homozygous lines. Actin gene (5’-TGTAGTCTGGGTAGTTAGT-3’ and 5’-GGAAGCTTCAGTCTTCTC-3’) was used as an internal control. No OsHKT1;5 transcript was present in this line (Supplemental Fig. S8B).

osmgt1 osmgt1;5 double-knockout mutants were generated by crossing homozygous osmgt1-1 (NF0595, Nipponbare background) with osmgt1-5 (Dongjing background). F1 heterozygote and F2 homozygous F1 plants were isolated by PCR genotyping (Supplemental Fig. S8C). The osmgt1 homozygous line was screened by PCR using specific primers (OsMGT1-F, 5’-AACCAGCATCTAAAAGTTTTCAC-3’ and OsMGT1-R, 5’-ATCTAGATATATGCTCCTCCACA-3’). To screen the lines for osmgt1 homozygous line was screened by PCR using specific primers (OsHKT1;5-F, 5’-GCCGCTTGCACACAGATGTAAGTGG-3’ and OsHKT1;5-R, 5’-GCTCACTACCTCAACCACCA-3’). The primer sequences (5’-GAGGTAGTAGGGCTTTACTTGTACAG-3’ and 5’-CGGACTAGTGCCGCTTTACTTGTACAG-3’) were used as an internal control. Normalized relative expression was calculated by the ΔΔCt method.

Phenotypic Analysis and Elemental Determination

To compare the sensitivity to salt, high Mg, and Cd toxicity, seedlings of both wild-type and two OsMGT1 knockout lines were exposed to a nutrient solution containing 50 mM NaCl, 10 mM MgCl$_2$, or 5 μM CdCl$_2$. After 12 d, individual plant was photographed, and leaves were separated and photographed. Both roots and shoots were sampled after the roots were washed with 5 mM CaCl$_2$ for three times to remove the apoplastics.

To investigate the Na accumulation in shoots, rice seedlings were exposed to a nutrient solution containing 50 mM NaCl for 3 d. The leaf blades 3 to 7 (from old to young) were separately sampled. Leaves 1 and 2 were too small to be sampled. Xylem sap was collected from the wild-type and the knockout. Roots exposed to a nutrient solution containing 10 mM NaCl for 24 h. The shoots (2 cm above the roots) were excised with a razor, and then the xylem sap was collected with a micropipette for 1 h after decapsulation of the shoots.

To investigate the sensitivity to osmgt1 osmgt1;5 double-knockout mutant to salt stress, the F3 progeny of wild type, single osmgt1 and osmgt1;5 mutants, and double mutants osmgt1 osmgt1;5 to a nutrient solution containing 0.02, 0.2, or 2 mM MgCl$_2$ in the presence of 50 mM NaCl for 7 to 12 d. Both roots and shoots were sampled for mineral analysis as described below.

To investigate the sensitivity of osmgt1 osmgt1;5, double-knockout mutant to salt stress, the F3 progeny of wild type, single osmgt1 and osmgt1;5 mutants, and double mutants showing the same genetic background were exposed to a nutrient solution containing 0 or 50 mM NaCl. After 7 d exposure, both the roots and shoots were sampled for analysis.

To harvest the roots and shoots were dried at 70°C for 2 d and then subjected to digestion with concentrated HNO$_3$. The metal concentration in the digested solution and xylem sap was determined by inductively coupled plasma-mass spectrometry (ICP-MS 7700x; Agilent Technologies). The Na content was calculated based on Na concentration and dry weight.

RNA Isolation and Expression Analysis

For time-response expression analysis, 14-d-old rice seedlings (cv. Nipponbare) were exposed to a 0.5× Kimura B solution containing 50 mM NaCl for different hours, and the roots and shoots were sampled for RNA extraction. For dose-dependent expression, rice seedlings were exposed to a 0.5× Kimura B solution containing different concentrations of NaCl for 6 h, and the roots were sampled for RNA extraction. For root spatial expression, different root segments (0–5, 5–10, and 10–20 mm from root tips) were excised after the roots exposed 0 or 50 mM NaCl for 24 h. To compare the salt tolerance genes expression between wild type and osmgt1 mutants, rice seedlings of both wild type and osmgt1 mutants were exposed to a 0.5× Kimura B solution with or without 50 mM of NaCl for 24 h, and the roots were sampled for RNA extraction.

Total RNA from rice roots and shoots was extracted using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using a ReverTra Ace qPCR RT Master Mix kit (TOYOBO) following the manufacturer’s instructions.

For gene expression level, the sensitivity of rice seedlings was determined by real-time RT-PCR using Thunderbird SYBR qPCR Mix (TOYOBO) on Mastercycler ep realplex (Eppendorf). The primers used were 5’-GCCGCGTTACGAGATTAGGG-3’ and 5’-CGCCGTATTCACGAGATGTAAGG-3’ for OsMGT1; 5’-TAACTACACACATCTGCGG-3’ and 5’-CTAACTGACGGATGTCCG-3’ for OsHKT1;5; 5’-CTGGAAACAGTGATCCAGAG-3’ and 5’-AGATCCAAAGGCGCCCTCCAAA-3’ for OsSOS1. Actin was used as an internal control. Normalized relative expression was calculated by the ΔΔCt method.

Tissue Specificity of Expression

The native 2.5-kb promoter sequence of OsMGT1 was linked to gfp gene by overlap PCR. The amplified PCR product was inserted into pZPF2H-lac (with NcoI terminus) using Atp1 and Spel to create the OsMGT1 promoter-GFP construct. The construct was introduced into the calluses of rice (cv. Nipponbare) via Agrobacterium tumefaciens-mediated transformation (Hiei et al., 1994). The primer sequences (5’-AATGGCCGATGCCCAGAC-3’ and 5’-CCGACCTATGCGCCCTTACTTCTGAC-3’) were used for amplification and introduction of the Atp1 and Spel restriction sites. Different organs and tissues were sampled for the immunostaining using an antibody against GFP (A11222, Molecular Probes) as described previously (Yamaji and Ma, 2007). For OsMGT1-responsive assay, root segments (20 mm from root tips) were sampled after the roots exposed to 0 or 50 mM NaCl for 24 h. Fluorescence of the secondary antibody (Alexa Fluor 550 goat anti-rabbit IgG; Molecular Probes) was observed with a confocal laser-scanning microscope (LSM700; Carl Zeiss).
OsHKT1;5 Expression Construct and TEVC Experiments Using Xenopus laevis Oocytes

The coding region of OsHKT1;5 cDNA was isolated from a japonica rice cultivar Nipponbare based on the sequence information at GenBank. The isolated cDNA was further amplified by the primers harboring the 5′/3′ site: Fw, 5′-AAGATCATGATGCTGACCCATA-3′ and Rev, 5′-CATGAGATCTTTATCTCTTCCATGCTGACCA-3′ using the Phusion high-fidelity DNA polymerase (New England Biolabs). The sequence of the amplified fragment was checked and then subcloned at the 5′/3′ site of the pXJG-ev1 vector (Suzuki et al., 2016). OsHKT1;5:DNA was transcribed using a mMESSAGE mMACHINE in vitro transcription kit (Ambion). TEVC experiments using oocytes of *X. laevis* frogs were performed as described previously (Suzuki et al., 2016). In brief, water or 12.5 ng of OsHKT1;5 cRNA was injected into oocytes and incubated at 18°C for 1 d. TEVC experiments were performed using an AxoClamp 900A amplifier (Molecular Devices), an Axon Instruments Digidata 1440A and pCLAMP 10 (Molecular Devices). Oocytes were bathed in a solution containing 2 mM Na-Glu, 1.8 mM or 18 mM CaCl2, 10 mM MES-1,3-bis (tris(hydroxymethyl)methylamino) propane, 180 mM t-mannitol (pH 5.5); with BicTrisPropane, and 0.1 mM, 1 mM, 10 mM MgCl2. The osmolarity of each solution was adjusted to 220 to 240 mosmol kg−1.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB731703 for OsMGT1 and AK108663 for OsHKT1;5.

**Supplemental Data**

The following supplemental materials are available.

Supplemental Figure S1. Sensitivity of OsMGT1 knockout lines to osmotic stress and water deficit.

Supplemental Figure S2. Sensitivity of OsMGT1 knockout lines to Mg and Cd toxicity.

Supplemental Figure S3. Comparison of Mg and K in the shoots, roots, and xylem sap of wild-type rice and knockout lines of OsMGT1.

Supplemental Figure S4. Gene expression of OsHKT1;4, OsHKT1;5, and OsSOS1 in wild-type and OsMGT1 knockout lines.

Supplemental Figure S5. Regulation of OsHKT1;5-mediated Na+ transport by 0.1 or 1.0 mM Mg2+.

Supplemental Figure S6. Regulation of OsHKT1;5-mediated Na+ transport by 1.8 or 18 mM Ca2+.

Supplemental Figure S7. Effect of high Mg2+ supply on salt sensitivity in osbhlk1.5 and osmgt1-1 osbhlk1.5 double mutant.

Supplemental Figure S8. Isolation of osmgt1-1 osbhlk1.5 double-knockout rice mutants.

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