

OsHAK4 functions in retrieving sodium from the phloem at the reproductive stage of rice

Jing Che^{1,2,3} , Naoki Yamaji², Shao Fei Wang^{1,3}, Yue Xia^{1,3}, Shun Ying Yang¹, Yan Hua Su^{1,3}, Ren Fang Shen^{1,3} and Jian Feng Ma^{2,*} 

¹Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China,

²Institute of Plant Science and Resources, Okayama University, Chuo 2-20-1, Kurashiki 710-0046, Japan, and

³University of Chinese Academy of Sciences, Beijing 100049, China

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*For correspondence (e-mail maj@rib.okayama-u.ac.jp).

SUMMARY

Soil salinity significantly limits rice productivity, but it is poorly understood how excess sodium (Na^+) is delivered to the grains at the reproductive stage. Here, we functionally characterized *OsHAK4*, a member of the clade IV HAK/KUP/KT transporter subfamily in rice. *OsHAK4* was localized to the plasma membrane and exhibited influx transport activity for Na^+ , but not for K^+ . Analysis of organ- and growth stage-dependent expression patterns showed that very low expression levels of *OsHAK4* were detected at the vegetative growth stage, but its high expression in uppermost node I, peduncle, and rachis was found at the reproductive stage. Immunostaining indicated *OsHAK4* localization in the phloem region of node I, peduncle, and rachis. Knockout of *OsHAK4* did not affect the growth and Na^+ accumulation at the vegetative stage. However, at the reproductive stage, the *hak4* mutants accumulated higher Na^+ in the peduncle, rachis, husk, and brown rice compared to the wild-type rice. Element imaging revealed higher Na^+ accumulation at the phloem region of the peduncle in the mutants. These results indicate that *OsHAK4* plays a crucial role in retrieving Na^+ from the phloem in the upper nodes, peduncle, and rachis, thereby preventing Na^+ distribution to the grains at the reproductive stage of rice.

Keywords: rice, *Oryza sativa*, salinity, HAK transporter, phloem unloading, node.

INTRODUCTION

Soil salinity is a major abiotic stressor that threatens crop growth and yield (Ismail & Horie, 2017; Liu et al., 2022; Munns & Tester, 2008). Excess salts (mainly NaCl) in saline soil cause both osmotic stress and ionic stress (Liu et al., 2022; van Zelm et al., 2020; Zhao et al., 2020; Zhou et al., 2024). Osmotic stress reduces water uptake by the roots, while over-accumulation of Na ion in plant tissues disturbs K homeostasis and various metabolic reactions (Wu, 2018; Raddatz et al., 2020; van Zelm et al., 2020). Especially, Na^+ accumulation in photosynthetic tissues is detrimental to crops, reducing photosynthetic carbon assimilation, eventually decreasing crop production (Atta et al., 2023; van Zelm et al., 2020; Zhao et al., 2020). Therefore, preventing Na^+ accumulation in plant tissues is very important to cope with soil salinity.

Transport of Na^+ from soil to various plant organs and tissues requires various transporters. Numerous transporters have been identified that mediate uptake and

exclusion by the roots, vacuolar sequestration, root-to-shoot translocation, xylem loading and unloading, phloem loading of Na^+ in different plant species (Adams & Shin, 2014; El Mahi et al., 2019; Ismail & Horie, 2017; Kronzucker & Britto, 2011; Ren et al., 2005; Wang et al., 2020; Zhang et al., 2019; Zhao et al., 2020). Several transporters belonging to different transporter families are involved in Na^+ uptake, which include glutamate receptor-like (GLR), cyclic nucleotide-gated channel (CNGC), high-affinity K^+ transporter 2 (HKT2), high-affinity potassium transporter 5 (HAK5), low-affinity cation transporter 1 (LCT1), and plasma membrane intrinsic protein 2;1 (PIP2;1) aquaporin (Byrt et al., 2017; Demidchik & Maathuis, 2007; Lan et al., 2010; Obata et al., 2007; Schachtman et al., 1997; Tapken et al., 2013). Exclusion of Na^+ from the roots is mediated by the salt overly sensitive 1 (SOS1) Na^+/H^+ exchanger (El Mahi et al., 2019; Shi et al., 2002; Zhao et al., 2020). Once Na^+ enters root cells, some of it is sequestered into the vacuoles by tonoplast-localized Na^+/H^+ exchangers in the NHX family

(Bassil & Blumwald, 2014), while the remaining part is released to the xylem by non-selective cation channel (NSCC), SOS1, cation-chloride cotransporters (CCC), HKT2 (Colmenero-Flores et al., 2007; Demidchik & Maathuis, 2007; Lan et al., 2010; Shi et al., 2002; Zhao et al., 2020). Na⁺ withdrawal from the xylem is achieved by HKT1 (Maser et al., 2002; Munns et al., 2012; Ren et al., 2005). In leaves, HKT1, SOS1, and NHX also play similar roles as in the roots (Bassil & Blumwald, 2014; van Zelm et al., 2020; Wu, 2018). Among them, SOS1, NHX, and HKT have been associated with salt tolerance because they contribute to lowering shoot Na⁺ accumulation.

Rice (*Oryza sativa* L.) is a staple food for half of the world's population, while it is relatively sensitive to salt stress compared with other cereal crops such as barley and wheat, although natural variations in salt tolerance exist (Munns & Tester, 2008). Since rice is also cultivated in salt-affected soils such as saline soils in coastal tropical zones and inland, breeding salt-tolerant rice cultivars to boost yield in these areas is of paramount importance (Ismail & Horie, 2017). Several genes involved in salt tolerance in rice have been identified. For example, some members belonging to HKT family including OshKT1;1, OshKT1;4, and OshKT1;5 contribute to salt tolerance by facilitating Na⁺ exclusion from leaves through withdrawal of Na⁺ from the xylem (Ren et al., 2005; Suzuki et al., 2016; Wang et al., 2015), resulting in decreased Na⁺ accumulation in growth active tissues. OshKT1;1 and OshKT1;5 are also involved in Na⁺ exclusion in the phloem, preventing Na⁺ transfer to young leaves (Kobayashi et al., 2017; Wang et al., 2015). Furthermore, OshKT1;1 is regulated by the transcription factor OsMYBc (Wang et al., 2015), which is ubiquitinated by OsMSRFP, an E3 ligase to fine-tune OshKT1;1 transcription (Xiao et al., 2022).

At the reproductive growth stage of rice, Na⁺ is transported to reproductive organs including flowers and grains, ultimately affecting grain yield. In Arabidopsis, it was reported that AtHKT1;1 regulated Na⁺ distribution and influenced seed production or plant fertility (An et al., 2017; Uchiyama et al., 2023), however, less is known about how Na⁺ is delivered to these reproductive organs in rice. Recently, nodes have been demonstrated to be a hub for the allocation of mineral elements to grains at the reproductive stage in rice (Yamaji & Ma, 2014, 2017). Especially, the uppermost node, node I, which is connected to the flag leaf and panicles, plays a crucial role in the allocation of mineral elements to the grains. Various transporters for mineral elements have been reported to be involved in this allocation process (Huang et al., 2024; Yamaji & Ma, 2014, 2017). Our previous transcriptome analysis revealed that *OshAK4* showed high expression level in the node I (Yamaji et al., 2013), but its exact role in ion allocation remains unclear.

OshAK4 belongs to the HAK/KUP/KT (high-affinity K⁺/K⁺ uptake/K⁺ transporter) family (Amrutha et al., 2007).

Several members of this family have been functionally characterized and linked to salt tolerance. For example, ZmHAK4 in maize functions as a high-affinity Na⁺-selective transporter, promoting shoot Na⁺ exclusion likely by retrieving Na⁺ from the xylem vessels (Zhang et al., 2019). SIHAK20 in tomato, an ortholog of ZmHAK4, transports both Na⁺ and K⁺, is involved in the loading of Na⁺ and K⁺ from parenchyma cells into the xylem in roots. Knockout of *SIHAK20* increases Na⁺ concentrations in roots and makes tomato plants more sensitive to salinity, while over-expression of *SIHAK20* triggers the opposite phenotype (Wang et al., 2020). Through detailed functional characterization of *OshAK4* in the present study, we found that unlike other reported HAK members, *OshAK4* plays a crucial role in retrieving Na⁺ from the phloem in the node I, peduncle and rachis, thereby preventing Na⁺ distribution to the grains at the reproductive stage of rice.

RESULTS

Cloning of *OshAK4* and phylogenetic analysis

Based on the sequence information of *OshAK4* (Os08g0466200) from the database (<http://rapdb.dna.affrc.go.jp/>), we amplified its ORF by using the cDNA of *Japonica* rice cultivar Nipponbare. The sequence obtained was identical to that in the database. The ORF is 2094 bp in length, encoding a protein with 697 amino acids. *OshAK4* shares a sequence identity of 78.9 and 64.0%, with ZmHAK4 and SIHAK20, respectively (Figure S1). The plant HAK family can be phylogenetically classified into four major clades, and *OshAK4* as well as ZmHAK4 and SIHAK20 belongs to clade IV (Figure S1).

Transport activity of *OshAK4*

OshAK4 has been reported to transport Na⁺ using yeast mutant (Zhang et al., 2019). To further characterize this transporter, we employed electrophysiological system for the transport activity assay using *Xenopus laevis* oocyte. A “stable recording strategy” was applied allowing the recordings of reversible development of Na⁺ currents at −80 mV in response to the introduction/withdrawal of the permeation ions (Hao et al., 2020a; Hao et al., 2020b; Figure 1A). When Na⁺ was introduced into the external solution, an inward current was rapidly activated only in the *OshAK4*-expressing oocyte but not in the H₂O-injected oocytes as a negative control (Figure 1B). Further analysis of the current–voltage relationship indicated that *OshAK4* mainly mediated inward Na⁺ uptake currents of large amplitude, showing clear voltage dependency (Figure 1C). In contrast, water-injected control oocytes exhibited small background currents under the same conditions (Figure 1C). Additionally, the inward Na⁺ uptake currents increased as the membrane potential increased (Figure 1C).

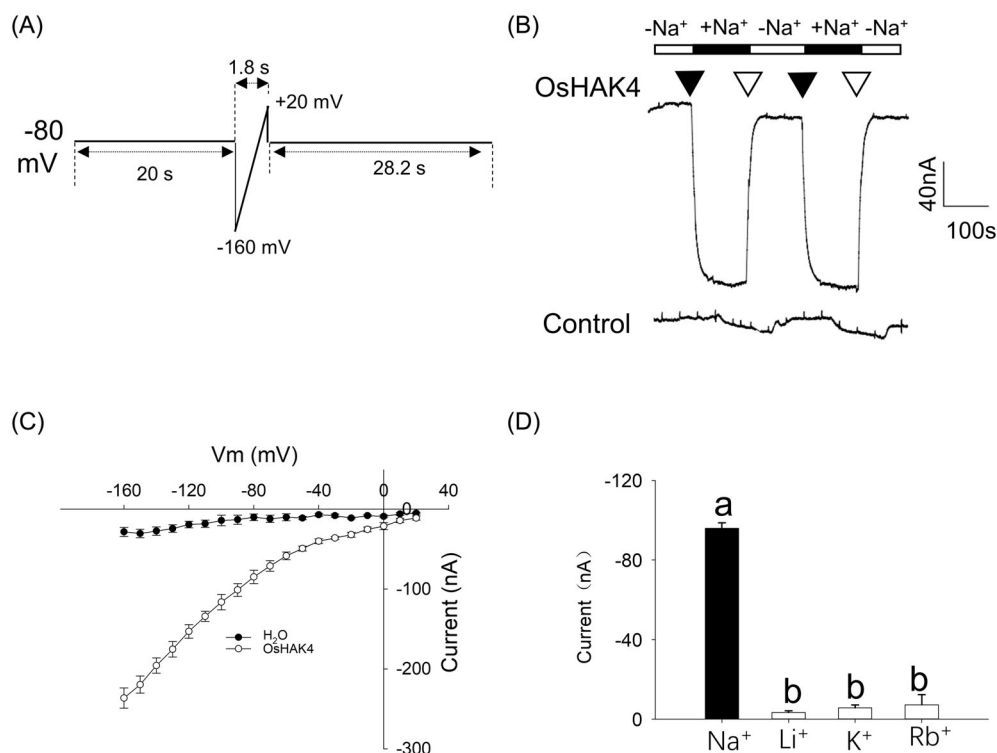


Figure 1. OSHAK4 mediates the uptake of Na⁺.

(A) Schematic illustration of the recording protocol used for the voltage-clamp measurements of OSHAK4-induced currents in *Xenopus* oocytes. A 1.8-sec voltage ramp ranging from -160 to $+20$ mV with an extended holding period at -80 mV for 48.2 sec was applied continuously during the experiment. (B) Representative recordings showing the current responses of oocytes injected with OSHAK4 (upper panel) or an equal volume of water (control, lower panel). The introduction (▲) or withdrawal (▽) of 100 mM Na⁺ is indicated. (C) Current–voltage relationship of OSHAK4-mediated Na⁺ uptake currents in oocytes. (D) Selectivity of OSHAK4 for different cation chloride salts (100 mM Na⁺, Li⁺, K⁺, and Rb⁺). Data were measured at -80 mV. Data are means $\pm$ SE ($n = 3$). Statistical results were obtained using one-way ANOVA and Student's *t*-test with SPSS 20.0 software.

We also examined pH-dependence of OSHAK4-mediated Na⁺ transport activity. At the pHs ranging from 5.4 to 7.4, the current was kept at a similar level (Figure S2).

Selectivity of OSHAK4 for other monovalent cations was also investigated. OSHAK4-dependent currents were recorded by bathing OSHAK4 cRNA-injected oocytes in solutions containing different cation chloride salts (Na⁺, Li⁺, K⁺, and Rb⁺). Among the four monovalent cation salts tested, NaCl elicited the largest inward currents, but other ions elicited much smaller currents (Figure 1D). These results indicate that OSHAK4 is a Na⁺-selective transporter.

OSHAK4 permeability to Na⁺ was further investigated by analyzing the dependency of the current magnitude on substrate concentrations. The Na⁺-induced current amplitude rapidly increased when the concentration of Na⁺ in the bath solution increased (Figure 2A). Further kinetic analysis showed that the uptake of Na⁺ could be described as biphasic behavior. The saturable Na⁺ uptake activity of OSHAK4 in the low concentration range of Na⁺ (5 mM and below) fitted to the Michaelis–Menten equation ($K_m = 0.18$ mM) (Figure 2B), while there was a linear response of OSHAK4 mediated currents in the high concentration range of Na⁺ (10–100 mM) (Figure 2B).

Subcellular localization of OSHAK4

The subcellular localization of OSHAK4 was observed by transiently expressing GFP-OSHAK4 fusion or GFP alone as a negative control in both rice leaf sheath protoplasts and onion (*Allium cepa* L.) epidermal cells. GFP alone showed signals in the cytosol and nucleus in the rice protoplasts and onion epidermal cells (Figure 3A–D; Figure S2A–D), which was well merged with the signal of DsRed, a marker of cytosol and nucleus (Figure S3A–D). However, in cells expressing the GFP-OSHAK4, the signal was observed at the cell periphery outward from the chloroplast in rice protoplast (Figure 3E–H). Furthermore, when expressed in onion epidermal cell, the fusion protein localized to the outer layer of the cell, which was not merged with DsRed (Figure S3E–H). All these results indicate that OSHAK4 is localized to the plasma membrane.

Organ- and growth stage-dependent expression patterns of OSHAK4

We investigated the expression pattern of OSHAK4 in different organs sampled at various growth stages of rice. OSHAK4 showed very low or no expression in all organs

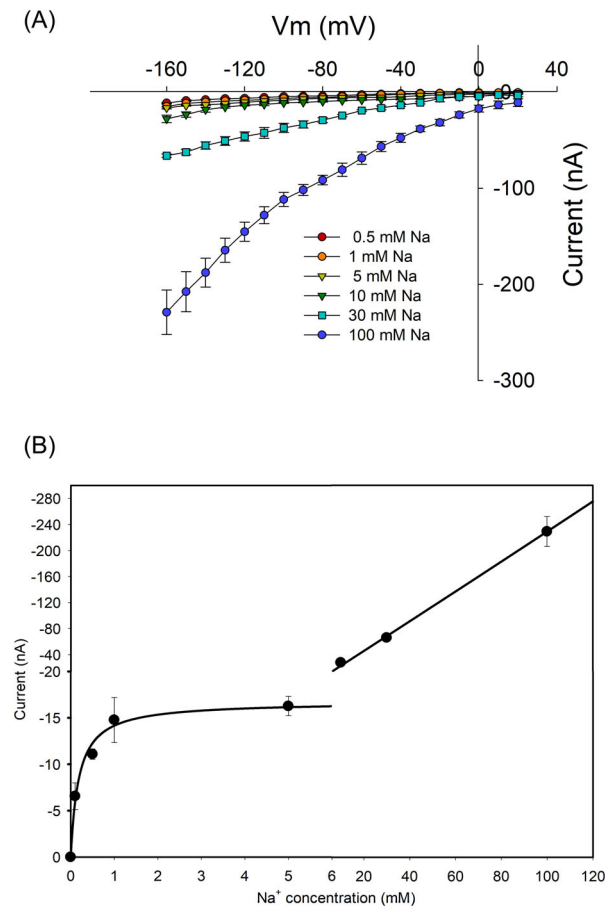


Figure 2. Biphasic behavior of *OsHAK4*-mediated Na⁺ uptake. (A) Current–voltage relationships of *OsHAK4*-mediated currents in response to Na⁺ concentrations (0.5, 1, 5, 10, 30, and 100 mM). (B) Kinetics of Na⁺ uptake activity of *OsHAK4* at different concentration ranges of Na⁺ (0.1–100 mM). The currents were measured at −160 mV. Data were means ± SE ($n = 3$). Statistical results were obtained using one-way ANOVA and Student's *t*-test with SPSS 20.0 software.

tested at the vegetative growth stage (Figure 4A). However, at the reproductive stage including booting, flowering, and grain filling stages, high expression was found in the upper nodes including node I and node II, peduncle, and rachis (Figure 4A). We also investigated the expression pattern of *OsHAK17*, the closest homolog of *OsHAK4*. In contrast to *OsHAK4*, *OsHAK17* was highly expressed in the leaf sheath and blade at all growth stages (Figure S4).

The response of *OsHAK4* expression to Na⁺ supply and K⁺-deficiency was also investigated in different organs sampled at the grain filling stage. The expression of *OsHAK4* was neither affected by Na⁺ supply nor by K⁺ deficiency in various organs tested, indicating its constitutive expression (Figure 4C).

Tissue-specific localization of *OsHAK4*

To investigate the tissue-specificity of *OsHAK4* expression, we generated transgenic rice plants expressing GFP under the control of the *OsHAK4* promoter. Based on the

expression pattern of *OsHAK4* (Figure 4), we observed its localization in the node I, peduncle and rachis sampled at the reproductive stage. Immunostaining with a GFP antibody revealed the presence of the signal in the phloem region of both enlarged vascular bundle (EVB) and diffuse vascular bundle (DVB) of node I, with a stronger signal in the phloem region of the DVB (Figure 5A,B). In the peduncle and rachis, the signal was similarly restricted to the phloem region (Figure 5D–E,G). No signal was detected in the node I, peduncle, and rachis of the non-transgenic wild-type plants (Figure 5C,F,H), indicating the specificity of the antibody.

Phenotypic analysis of *OsHAK4* knockout lines at the vegetative growth stage

To investigate the physiological role of *OsHAK4* in rice, we generated knockout lines using CRISPR/Cas9-mediated targeted mutagenesis. We obtained two independent lines; *hak4-1 + a* and *hak4-2 + t*, each with a 1 bp insertion (A or T insertion) at two different targeted sites (Figure S5). The *hak4-1 + a* line resulted in premature stop codon at the position of 22 amino acid, while *hak4-2 + t* line resulted in a premature stop codon at the position of 91 amino acid of the *OsHAK4* protein.

When seedlings of both wild-type (WT) rice and *hak4* mutants were subjected to 10 mM or 50 mM NaCl for 14 days at the seedling stage, the growth of both the roots and shoots was inhibited by 50 mM NaCl supply compared to 10 mM NaCl in both the WT and mutants (Figure S6A, B). However, there was no difference in the growth between the WT and two mutants (Figure S6A,B). The concentration of Na⁺ as well as K⁺ in the roots and shoots also did not differ between the WT and mutants (Figure S6C,D). Further investigation of the organ-dependent Na⁺ concentration under 50 mM NaCl treatment revealed no difference in Na⁺ accumulation in different organs between the WT and mutants at the vegetative growth stage (Figure S6E).

Additionally, the concentration of Na⁺ in the xylem sap was compared between WT and mutants at low and high Na⁺ supply concentration at the seedling stage. At both Na⁺ conditions, no significant difference in the xylem sap Na⁺ concentration was found between WT and mutants (Figure S6F).

Phenotypic analysis of *OsHAK4* knockout lines at the reproductive growth stage

Next, to examine the role of *OsHAK4* at the reproductive growth stage, we grew WT and *hak4* mutants in soil amended with 1.1 g/kg Na⁺ until maturity. The growth of *hak4* mutants was comparable to that of WT (Figure S7A). The plant height, grain number per panicle, filled spikelet, 1000-grain weight, and grain weight per plant were similar between WT and mutants (Figure S7A–F). However, the panicle number was less in the mutant lines than in WT, especially for the *hak4-2 + t* mutant (Figure S7G). Analysis

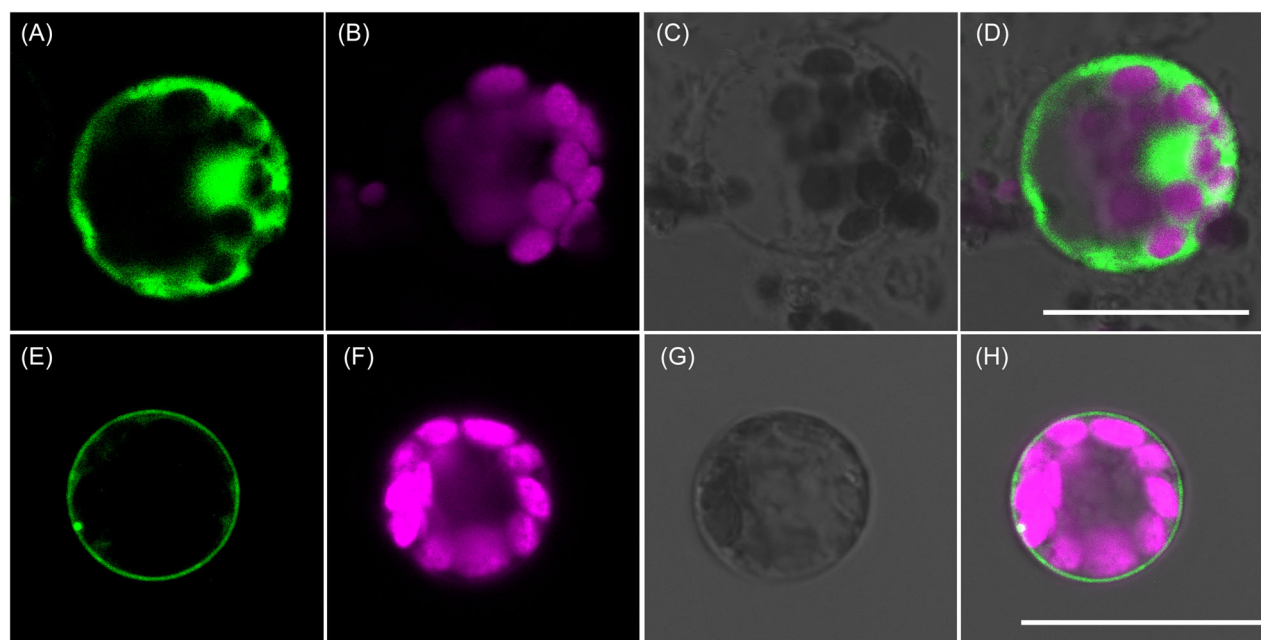


Figure 3. Subcellular localization of OsHAK4.

(A–H) Signal of GFP in rice protoplast. GFP alone (A–D) or GFP-OsHAK4 fusion (E–H) was transiently expressed in rice protoplast. GFP signal (A, E), chloroplast signal (B, F), bright field image (C, G), and a merged image (D, H) are shown. Scale bars = 25 μm .

of Na^+ and K^+ accumulation in different organs showed that the *hak4* mutants accumulated higher Na^+ in the peduncle, rachis, husk, and brown rice compared to WT (Figure 6A). By contrast, there was no difference in concentration of K^+ in different organs between WT and *hak4* mutants, except that *hak4-2 + t* mutant accumulated slightly lower K^+ in the flag leaf sheath and peduncle (Figure 6B).

Short-term distribution of Na^+ in different organs at the reproductive growth stage

To further investigate the role of OsHAK4 in Na^+ distribution at the reproductive growth stage, we performed a short-term experiment by exposing both WT and mutants grown hydroponically to 50 mM NaCl for 2 days at the flowering stage. The results showed that *hak4* mutants accumulated higher levels of Na^+ in the peduncle and panicle, but similar level in the node I, flag leaf blade, and sheath compared with WT (Figure 7A). Calculation of Na^+ distribution showed that more Na^+ was distributed to the peduncle and panicles, but less to the node I and flag leaf in the mutants (Figure 7B). In contrast, there was no difference in the concentration of K^+ and other mineral elements in different organs between WT and *hak4* mutants (Figure S8).

Tissue-specific deposition of Na^+ in the peduncle and flag leaf sheath

To observe tissue-specific deposition of Na^+ in the peduncle and flag leaf sheath, we applied laser ablation-inductively coupled plasma-mass spectrometry

(LA-ICP-MS). Different deposition pattern was detected in the WT and mutants. In the WT, Na^+ was mainly detected in the inner side of the flag leaf sheath (Figure 8A), whereas Na^+ was mainly deposited at the phloem region of the peduncle in the mutant (Figure 8B). As controls, the deposition of K^+ and Si was also compared between the WT and mutant. Unlike Na^+ , K^+ was detected in both the peduncle and flag leaf sheath (Figure 8C,D), with no difference in the deposition pattern between the WT and mutant. Si was mainly deposited in the peripheral tissues of the flag leaf sheath of both WT and mutant plants (Figure 8E,F), but no difference was observed between these lines. The superimposed image of the three elements clearly showed different deposition of Na^+ in the peduncle and flag leaf sheath between WT and mutant, whereas the deposition pattern of K^+ and Si was similar (Figure 8G,H).

DISCUSSION

OsHAK4 is a Na^+ -selective transporter

HAK forms a large transporter family, which primarily is involved in K^+ transport, with some members also transporting Na^+ (Benito et al., 2012; Takahashi et al., 2007; Wang et al., 2020; Zhang et al., 2019). For instance, PHAK5 in *Phragmites australis* and SIHAK20 can transport both Na^+ and K^+ , while ZmHAK4 exclusively transports Na^+ (Zhang et al., 2019). PpHAK13 from *Physcomitrella patens* is a high-affinity Na^+ transporter (Benito et al., 2012). LjKUP from *Lotus japonicus* complements K^+ uptake deficiency in

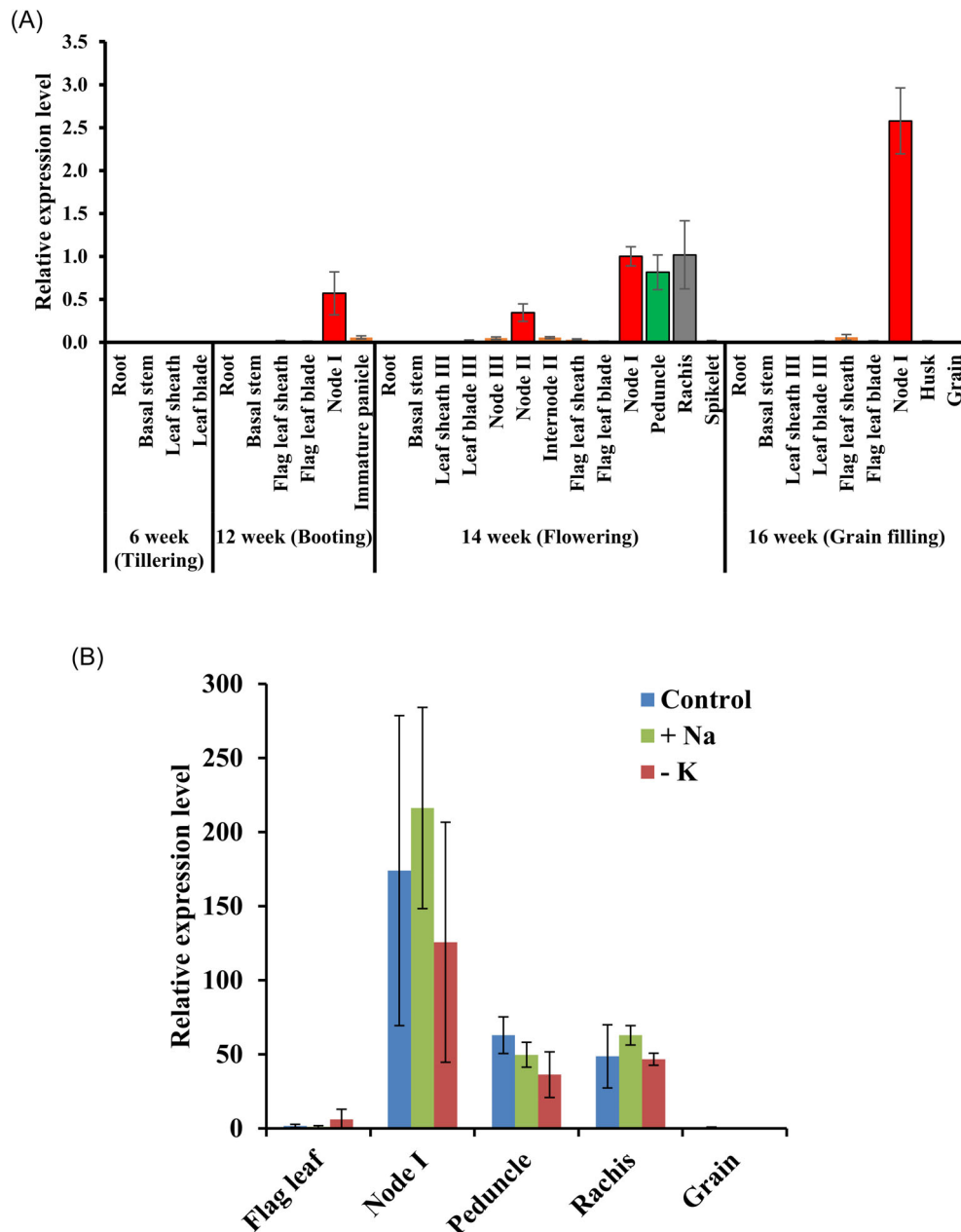


Figure 4. Expression patterns of *OsHAK4*.

(A) Expression pattern of *OsHAK4* in various organs at different growth stages. Rice was grown in a paddy field, and various organs were sampled at different growth stages.

(B) Response of *OsHAK4* expression to salt stress and K-deficiency at the grain filling stage. Rice was cultivated in a nutrient solution until grain filling and then transferred to a solution with or without K, or with 50 mM NaCl for 1 week. Different organs were sampled for RNA extraction. The expression level was determined by reverse transcription quantitative PCR. Expression level relative to node I at the flowering stage (A), and to flag leaf under the control condition (B) is shown. *HistoneH3* was used as an internal standard. Data are means $\pm$ SD of three biological replicates.

bacteria (Desbrosses et al., 2004). The rice genome contains 27 members of the HAK family (Amrutha et al., 2007, Figure S1), which are classified into four different clades (Amrutha et al., 2007; Gupta et al., 2008). Some of them have been functionally analyzed. For example, *OsHAK1*, *OsHAK5*, *OsHAK16*, and *OsHAK21*, all belonging to the clade I, have been shown to transport K^+ with varying

affinities and are involved in K^+ uptake and salt tolerance (Chen et al., 2015; Feng et al., 2019; Shen et al., 2015; Yang et al., 2014). *OsHAK9* belonging to clade II functions as a K^+ transporter and regulates seed germination under salt stress (Zeng et al., 2024). In clade III, *OsHAK12* is involved in retrieving Na^+ from xylem back to root tissues in response to salt stress Zhang et al., 2021), while *OsHAK18*

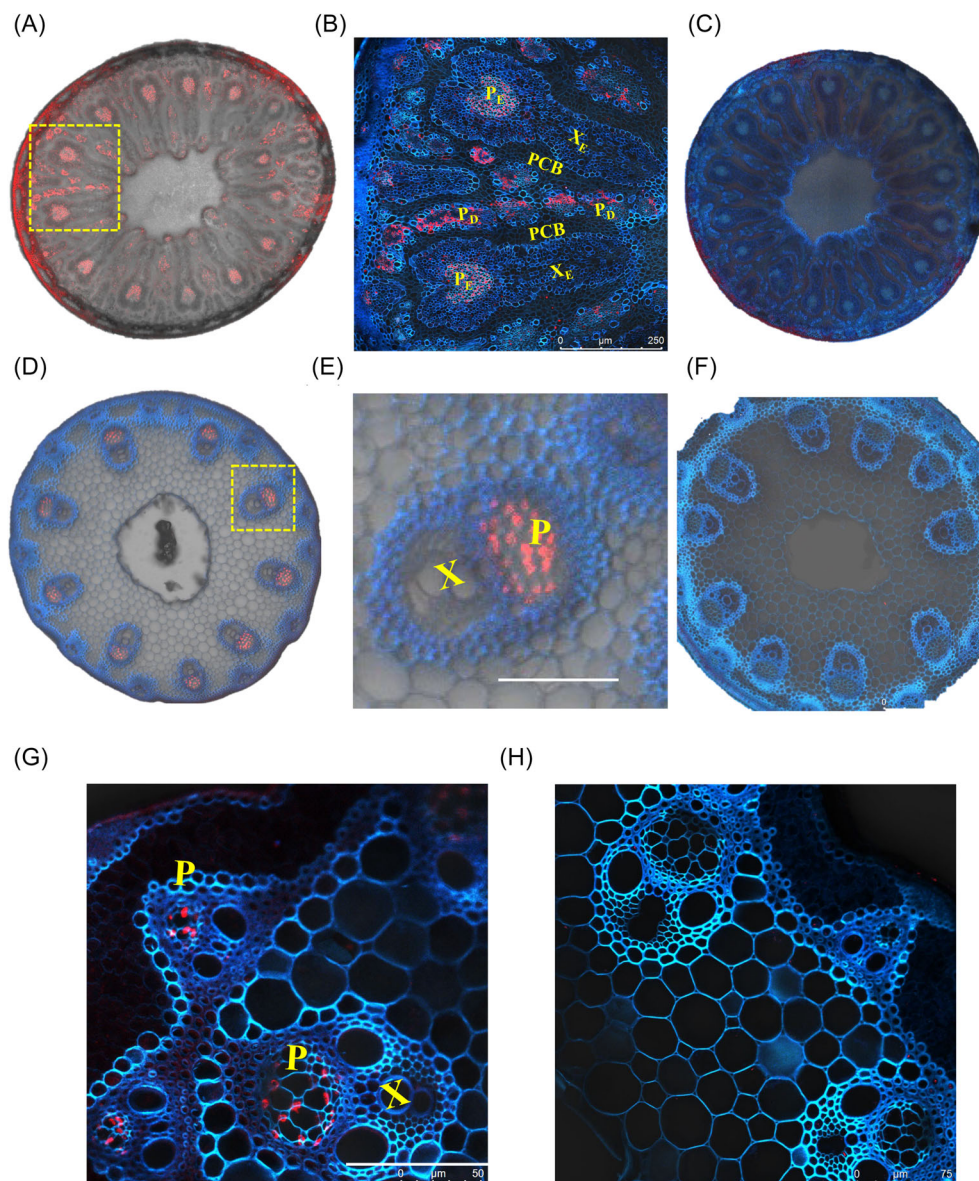


Figure 5. Tissue specificity of *OsHAK4* expression in different organs.

(A–H) Localization of *OsHAK4* in node I (A–C), peduncle (D–F), and rachis (G–H). Immunostaining with a GFP antibody was performed in transgenic rice carrying the *OsHAK4* promoter fused with GFP (A, B, D, E, G) or WT (C, F, H) as a negative control at flowering growth stage. Red color indicates the GFP antibody-specific signal. Blue color indicates cell wall autofluorescence. Yellow dotted boxes in A and C represent regions magnified in B and D, respectively. Xylem (X), phloem (P), xylem area of enlarged vascular bundle (X_E), parenchyma cell bridge (PCB), phloem area of diffuse vascular bundles (P_D), and phloem area of enlarged vascular bundle (P_E) are shown. Scale bar is 100 μ m.

mediates shoot-to-root circulation of Na^+ and K^+ (Peng et al., 2023), phloem K^+ loading and distribution (Shen et al., 2023). However, the role of HAK members in clade IV in rice has been less investigated. In this study, we identified that *OsHAK4* is a Na^+ -selective transporter. This conclusion is supported by evidence showing that *OsHAK4* only transports Na^+ but not K^+ , Rb^+ , and Li^+ when expressed in *Xenopus* oocytes (Figure 1). Difference in Na^+ concentration rather than K^+ concentration in peduncle, rachis, and grains between the WT and mutants further supports this conclusion. These results indicate that

OsHAK4 shows similar selectivity as other members belonging to the clade IV including *ZmHAK4*, *TaHAK4-A*, and *PpHAK13* from different plant species. However, the affinity for Na^+ seems to differ within clade IV members. *PhaHAK5* functions as a low-affinity Na^+ transporter, while *SIHAK20* is a high-affinity Na^+ transporter (with K_m values of 26.8 μ M for *SIHAK20*^{Hap1} or 40.1 μ M for *SIHAK20*^{Hap2}) (Takahashi et al., 2007; Wang et al., 2020). The K_m of *OsHAK4* was estimated to be 180 μ M at low Na^+ concentrations (5 mM and below), indicating that it functions as a high-affinity transporter for Na^+ (Figure 2). Interestingly, at

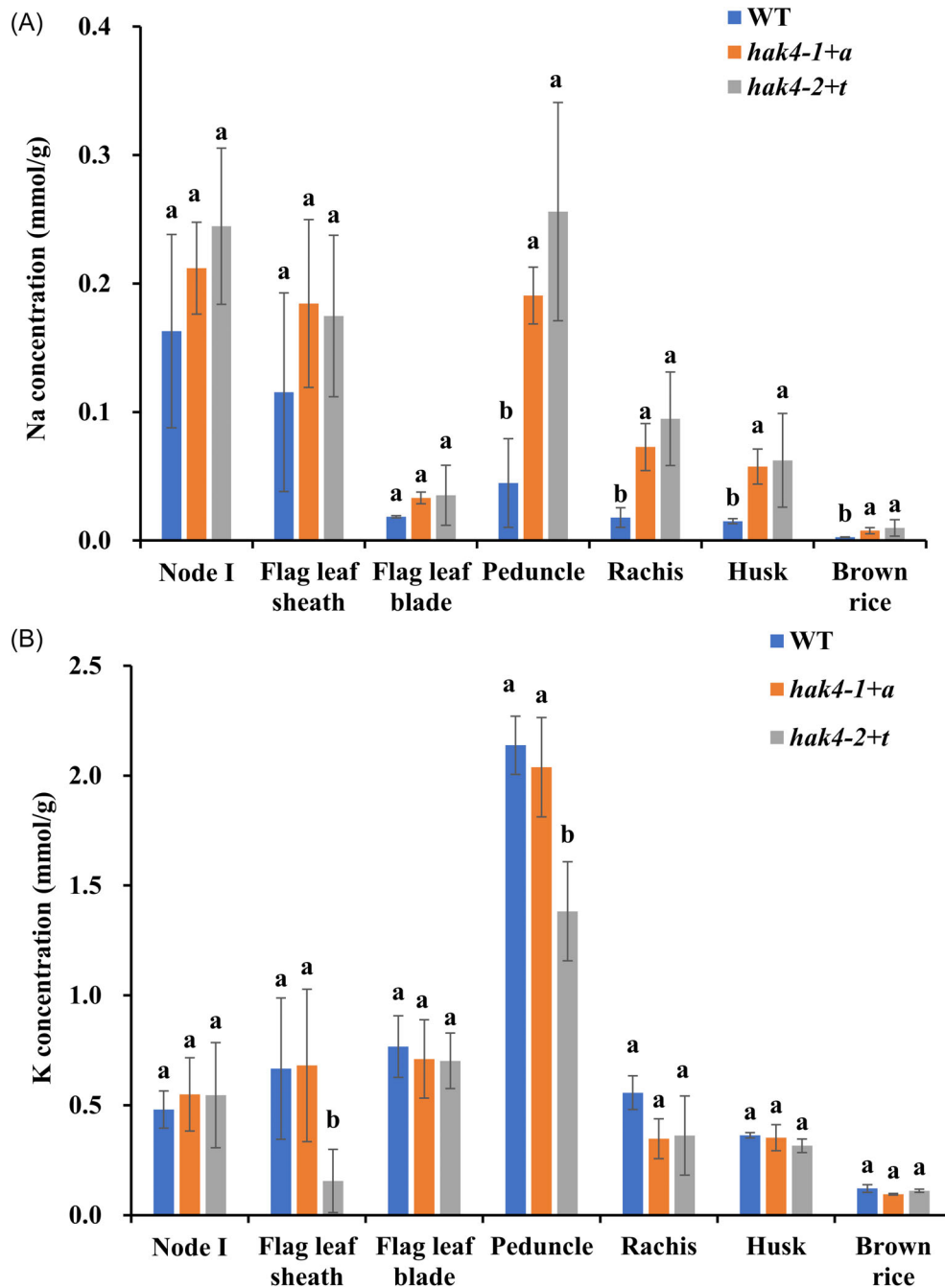


Figure 6. Concentration of Na⁺ (A) and K⁺ (B) in different organs in the wild-type rice (WT) and *hak4* mutants.

WT rice and two independent mutant lines were grown in pot soils containing Na⁺ for 4 months. Data represent the means $\pm$ SD ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between WT and *hak4* mutants in each organ using Duncan's test.

high Na⁺ concentrations (>10 mM Na⁺) (Figure 2), the uptake shifts to a linear response although its physiological roles in plants remain to be investigated.

Some HAK members showed pH-dependent activity. For example, a higher activity of ZmHAK4 for Na⁺ was observed at acidic pH than at an alkaline one (Zhang

et al., 2019). DmHAK5 has been reported to be a high-affinity K⁺ transporter driven by proton (Scherzer et al., 2015). However, we found that OsHAK4 does not show pH-dependence (Figure S2). Considering that OsHAK4 is localized at the phloem having a pH above 7.0 (Figure 5), this result might be reasonable.

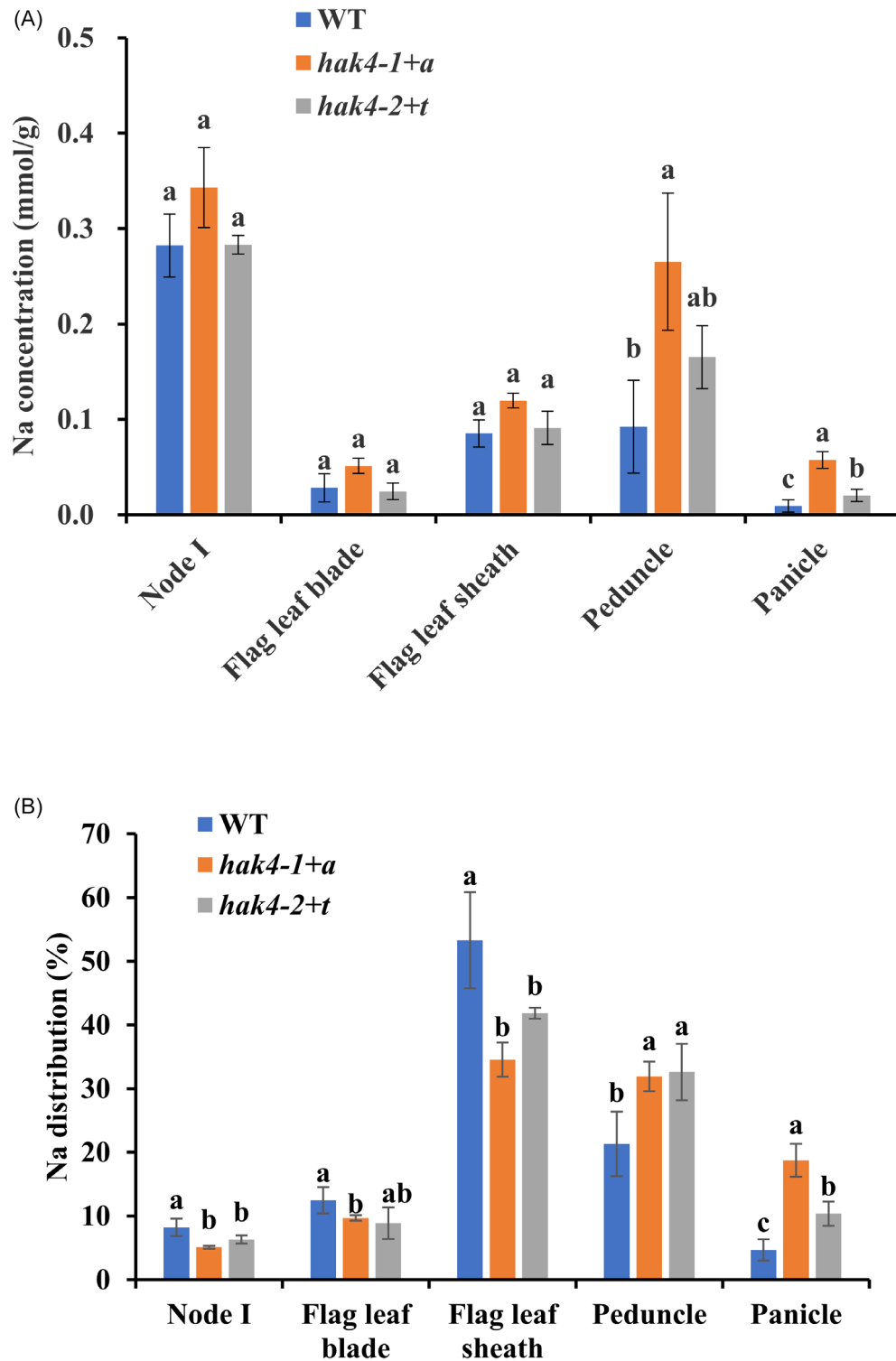


Figure 7. Short-term labeling experiment with NaCl.

(A, B) Concentration (A) and distribution (B) of Na^+ in different organs of wild-type (WT) rice and *hak4* mutants. Seedlings of WT and *hak4* mutant lines at the flowering stage were exposed to 50 mM NaCl for 2 days. Different organs, including node I, flag leaf blade, flag leaf sheath, peduncle, and panicle were separately harvested and subjected to Na^+ determination. Data represent the means $\pm$ SD ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between WT and *hak4* mutants in each organ using Tukey's test.

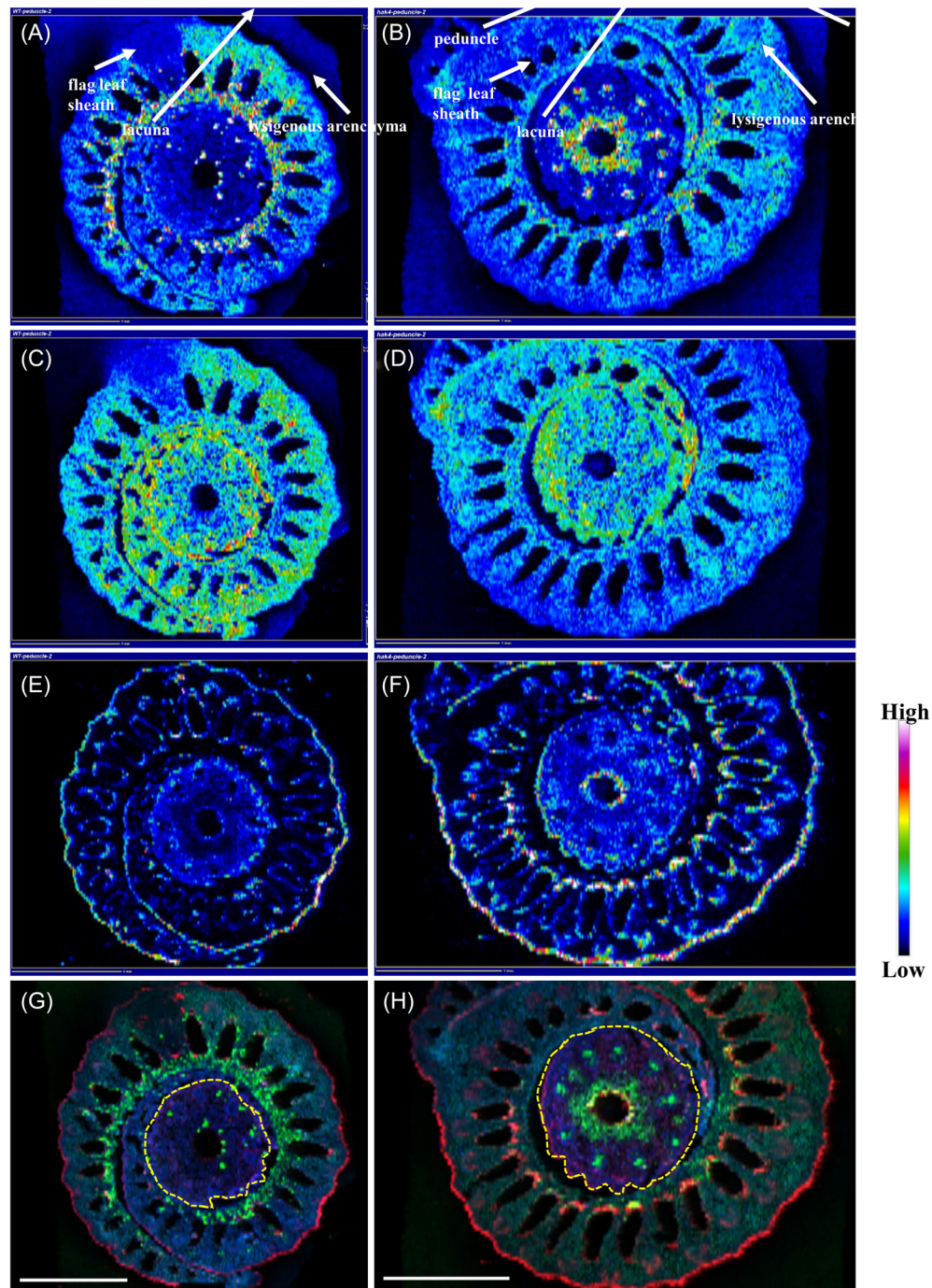


Figure 8. Deposition pattern of Na in the peduncle and flag leaf sheath.

(A–F) Mapping image of Na (A, B), K (C, D), and Si (E, F) in cross sections of peduncle and flag leaf sheath of wild-type (WT) rice (A, C, E, and G) and *hak4-1 + a* mutant (B, D, F, and H). Both WT rice and *hak4* mutant were grown in pot soil. At the flowering stage, 4 g NaCl per pot was added to the soil and peduncle including the flag leaf sheath of rice at the ripening stage was sampled for detection of Na deposition as well as K and Si as controls by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). (G and H) Superimposed image of three elements; Na (green), K (blue), and Si (red). The peduncle (inside the line) and flag leaf sheath (outside of the line) are separated by a yellow-dotted line. Flag leaf sheath, lysigenous arechyma, peduncle, and lacuna, phloem, xylem in the peduncle are shown. Scale bar is 1 mm.

Phloem-localized OSHAK4 is probably involved in excluding Na⁺ from the phloem at the reproductive growth stage

Through comprehensive functional analysis, we found that OSHAK4 exhibited a unique expression pattern, tissue-specific localization, and mutant phenotype, leading to its distinct role in Na⁺ accumulation from its homologs such as ZmHAK4. We found that knockout of *OshAK4* did not affect both the growth and Na⁺ accumulation at the vegetative stage (Figure S6). This is different from ZmHAK4, whose knockout lines accumulated higher Na⁺ in the shoots (Zhang et al., 2019). This difference could be attributed to their expression pattern and localization. Unlike ZmHAK4, which showed high expression in the root stele (Zhang et al., 2019), *OshAK4* expression level was very low in all organs at the vegetative growth stage (Figure 4A), suggesting that OSHAK4 plays a minor role in Na⁺ accumulation. This is supported by the fact that knockout of ZmHAK4 led to increased Na⁺ in the xylem sap (Zhang et al., 2019), whereas knockout of *OshAK4* did not affect xylem Na⁺ concentrations (Figure S5F). These findings indicate that unlike ZmHAK4, OSHAK4 is not participated in Na⁺ retrieval from xylem vessels. In a previous study (Wang et al., 2020), a double mutant *oshak4/17* exhibited higher sensitivity to salt stress at the vegetative stage although each single mutant phenotype was not examined. *OshAK17* is the closest homolog of *OshAK4* in clade IV (Figure S1), but it showed different expression pattern from that of *OshAK4* (Figure S5). The phenotype observed in the double mutant may result from the effect of *OshAK17* knockout although further investigation is required.

However, at the reproductive stage, knockout of *OshAK4* altered Na⁺ deposition (Figure 8); more Na⁺ was accumulated in the peduncle, rachis, and grains including husk and brown rice of the mutants (Figure 6). At the reproductive stage, after uptake by the roots, Na⁺ is translocated to node I before reaching the grain. At node I, Na⁺ undergoes distribution to different organs. A short-term experiment showed that more than 70% of total Na⁺ is distributed to the flag leaf (Figure 7), while the remaining part is distributed to other organs above the node I. *OshAK4* showed high expression in the upper nodes, especially in node I, and localized in the phloem region of both DVB and EVB (Figure 5A,B). Phloem is connected to the developing tissue such as grains, therefore exclusion of Na⁺ from the phloem sap will protect these organs to over-accumulate Na⁺. Our results indicate that OSHAK4 at the upper nodes is likely responsible for the unloading of Na⁺ from the phloem. This is supported by the finding that knockout of *OshAK4* resulted in increased Na⁺ distribution to the peduncle and panicles, but decreased Na⁺ distribution to the flag leaf sheath and node I (Figure 7B). This role of OSHAK4 serves to prevent the upward transfer of

sodium through the phloem to more sensitive tissues such as panicle in rice. In fact, *oshak4* mutants accumulated higher Na⁺ in brown rice compared with the WT (Figure 6A). LA-ICP-MS results showed that Na⁺ was mainly accumulated in the phloem region of the peduncle in the mutant, which was not observed in WT (Figure 8).

After passing through the node I, Na⁺ is further retrieved from the phloem by OSHAK4, which is localized at the phloem region of the peduncle and rachis, thereby averting its transfer to the panicles (Figure 5C–E). Therefore, loss of function of *OshAK4* decreased Na⁺ exclusion from the phloem, leading to Na⁺ accumulation in these organs (Figures 6A and 7A). Collectively, these findings imply that OSHAK4 functions in the recirculation of Na⁺ from the phloem of reproductive organs.

Different from other homologous genes such as ZmHAK4, knockout of *OshAK4* hardly affected the yield under salt stress (Figure S6A). One possible explanation is the presence of other transporters with similar functions. In fact, OsHKT1;1 and OsHKT1;5 are also localized in the phloem region in the leaves in addition to root stele and may play redundant roles in exclusion of Na⁺ from the phloem (Kobayashi et al., 2017; Ren et al., 2005; Wang et al., 2015).

In summary, our results indicate that OSHAK4 is a plasma membrane-localized Na⁺-selective transporter in rice, playing a crucial role in Na⁺ exclusion from the phloem, thereby preventing the transfer of Na⁺ to the panicles at the reproductive growth stage in rice.

EXPERIMENTAL PROCEDURES

Cloning the full length of *OshAK4*

To clone the full-length ORF of *OshAK4*, total RNA was extracted from rice node I (cv. Nipponbare) using an RNeasy Plant Mini Kit (Qiagen). The extracted RNA was then reverse-transcribed into cDNA using a ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO), following the manufacturer's protocol. The full-length ORF was amplified by PCR using primers listed in Table S1, designed based on a putative cDNA clone (Os08g0466200) from the Rice Annotation Project Database, with putative translational start and stop site. The amplified cDNA was then cloned into the pGEM[®]-T vector (Promega) and the sequence was confirmed using an ABI Prism 3130 sequence analyzer (Applied Biosystems).

Electrophysiological analysis of OSHAK4 in *Xenopus* oocytes

For *Xenopus* oocyte expression, the coding sequence of OSHAK4 was subcloned into the pT7TS vector (Biovector Inc. USA). One µg of linearized plasmid DNA was used for *in vitro* cRNA synthesis using a T7 mMESSAGE mMACHINE[®] Kit (AM1344, Thermo Fisher Scientific). Oocyte preparation and micro-injection were performed as previously described (Hao et al., 2020a, 2020b; Huang et al., 2021). Briefly, oocytes were surgically removed from female *Xenopus laevis* frogs, defolliculated with 1 mg/ml collagenase A (Roche) and rinsed. Healthy oocytes were selected and injected

with a Nano-liter 2000 micro-injector (WPI, Sarasota, Florida, USA). Each oocyte received 29.9 ng of *OsHAK4* cRNA in a volume of 59.8 μ l. Control oocytes were injected with the same volumes of H₂O. Oocytes were incubated at 19°C in ND96 solution composed of (in mM) 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, pH 7.4, and 50 μ g/mL gentamycin. For pH-dependence experiment, 4 mM MES was added and Tris was used to adjust the pH to 5.4, 6.4, and 7.4. Current recordings were applied 30 h after injection by two-electrode voltage-clamp technique supported by an AxoClamp 900A amplifier (Molecular Devices, San Jose, California, USA). The basal recording solution contained (in mM): 100 choline-chloride, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4 with Tris-base. For Na⁺ current measurement, NaCl of desired concentrations was added to the basal recording solution to replace the same strengths of choline-chloride, and the solution change was achieved through perfusion. For the analysis of ionic permeation spectrum other than NaCl, 100 mM NaCl in the recording solution was replaced by same concentration of LiCl, KCl or RbCl. A “stable recording strategy” (Hao et al., 2020a, 2020b) was applied allowing the recordings of reversible development of Na⁺ currents at –80 mV in response to the introduction/withdrawal of the permeation ions. The current amplitudes were captured simultaneously at both the peak and baseline levels of the current development using integrated voltage ramps. These ramps ranged from –160 to +20 mV and were completed in 1.8 sec. The pH-dependent transport activity was measured at –80 mV in the presence of 100 mM NaCl.

Stable electrophysiological measurements were collected from three independent experiments. The electrophysiological data were extracted with pClampfit 10.3 (Molecular Devices) and analyzed with SigmaPlot 12.5 software. Each mean value is derived from measurements taken from three oocytes.

Expression pattern analysis

To investigate the expression patterns of *OsHAK4* and *OsHAK17* in different organs at different growth stages in rice grown in a paddy field, cDNA previously prepared from rice (cv. Nipponbare, WT) were used (Yamaji et al., 2013). To examine the expression response to salt stress and K-deficiency, rice plants were cultivated in a nutrient solution until grain filling and then exposed to a solution with or without K, or with 50 mM NaCl for 1 week. The flag leaf, node I, peduncle, rachis, and grain were sampled for RNA extraction.

Total RNA extraction and cDNA conversion were performed as described above. Expression level was determined by RT-qPCR using a THUNDERBIRD™ qPCR mix (TOYOBO) or SsoFast EvaGreen Supermix (Bio-Rad) on real-time PCR machine CFX384 (Bio-Rad). *HistoneH3* was used as an internal control. Relative expression levels were calculated by the comparative Ct method. Three independent biological replicates were performed for each treatment. The primer sequences used are shown in Table S1.

Tissue and cellular specificity of *OsHAK4* expression

To investigate the tissue-specific expression of *OsHAK4*, transgenic rice lines carrying the *OsHAK4* promoter fused with GFP were generated. The promoter region of *OsHAK4* (2.1 kb) was amplified from rice genomic DNA using primers listed in the Table S1. The promoter fragment was then fused to the GFP fragment isolated from a pBluescript vector (Agilent). The fused construct was inserted into the pPZP2H-lac vector and subsequently transformed into calluses (cv. Nipponbare) via *Agrobacterium tumefaciens*-mediated transformation (Hiei et al., 1994). Immunostaining with an anti-GFP antibody was performed in the node I,

peduncle, and rachis of the transgenic rice harboring p*OsHAK4*-GFP and non-transgenic rice as a negative control as previously described (Yamaji & Ma, 2007).

Subcellular localization of *OsHAK4*

The subcellular localization of *OsHAK4* was examined by introducing *GFP-OsHAK4* fusion or *GFP* alone into rice protoplasts and onion (*Allium cepa* L.) epidermal cells. The ORF of *OsHAK4*, without or with the stop codon, was amplified by PCR from rice (cv. Nipponbare) node cDNA for *GFP-OsHAK4* fusion. The ORF was inserted between the cauliflower mosaic virus 35S promoter and GFP-NOS terminator in the pBluescript vector. The primers with the *BspEI* site used were listed in Table S1. Rice protoplasts were prepared from leaves of 2-week-old seedlings grown hydroponically. The polyethylene glycol method was used for transformation (Chen et al., 2006). The same fused genes were introduced into onion epidermal cells as described previously (Che et al., 2018). GFP fluorescence was observed by laser confocal microscopy (LSM700; Carl Zeiss). The excitation/emission wavelengths were 488 nm/498 to 540 nm for GFP, 640 nm/670 to 720 nm for chloroplast, and 560 nm/580 to 620 nm for DsRed.

Generation of *hak4* knockout lines by CRISPR/Cas9

The knockout lines of *OsHAK4* were generated by the CRISPR-Cas9 system (Mikami et al., 2015). A 20 bases upstream of the PAM motif was selected as candidate target sequence at two different positions using primers listed in Table S1 (Figure S2). Double-stranded target sequences were cloned into pU6gRNA vector by annealing the paired-single oligonucleotides at 95°C for 5 min and linearizing the vector with *BbsI*. OsU3-gYSA in pZDgRNA-Cas9ver.2_HPT was replaced by synthetic gRNA expression constructs using *Ascl* and *Pacl* sites. The derived construct was transformed into calluses (cv. Nipponbare) by *A. tumefaciens*-mediated transformation.

To genotype the resultant mutants, genomic DNA was extracted from leaves of transgenic lines. PCR amplifications were carried out using primer pairs flanking the designed target sites as listed in Table S1. The PCR products (approximately 500 bp) were sequenced directly using internal specific primers, of which the binding positions are desirably at approximately 200 bp upstream of the target sites. Two homologous knockout lines (*hak4-1 + a* and *hak4-2 + t*) without Cas9 were selected and the T3 generation was used in subsequent phenotypic analysis.

Physiological analysis of *hak4* mutant at different growth stages

Seeds of wild-type rice (*Oryza sativa* cv. Nipponbare, WT) and two homozygous mutants (*hak4-1 + a* and *hak4-2 + t*) were soaked in water at 30°C in the dark for 2 days and then transferred to a net floating on a 0.5 mM CaCl₂ solution. On day 7, seedlings were transferred to a 3.5-L plastic pot containing half-strength Kimura B solution. The nutrient solution contained the following macronutrients (mM): (NH₄)₂SO₄ (0.18), MgSO₄·7H₂O (0.27), KNO₃ (0.09), Ca(NO₃)₂·4H₂O (0.18) and KH₂PO₄ (0.09), and the micronutrients (μ M): FeSO₄ (2), MnCl₂·4H₂O (0.5), H₃BO₃ (3), (NH₄)₆MoO₇·24H₂O (1), ZnSO₄·7H₂O (0.4) and CuSO₄·5H₂O (0.2). The pH of this solution was adjusted to 5.6, and the nutrient solution was renewed every 2 days. Plants were grown in a closed greenhouse at 23–30°C under natural light.

For phenotyping at the vegetative growth stage, 16-day-old seedlings of *hak4* mutants and WT were exposed to a nutrient solution containing 10 mM or 50 mM NaCl. The solution was

renewed every 2 days. After a 2-week exposure, the roots, shoot basal region, and different leaves were sampled for growth analysis and mineral element determination as described below. Three replicates were conducted for each experiment.

For phenotypic analysis at the reproductive stage, we performed a soil culture with three biological replicates. 18-day-old seedlings grown hydroponically were transplanted to a 3.5-L plastic pot filled with paddy soil containing 1.1 g/kg Na⁺. Tap water was supplied daily. At the ripening stage (4 months later), the plants were harvested and separated into node I, flag leaf blade, flag leaf sheath, peduncle, rachis, husk, and brown rice for mineral element analysis as described below. Yield components were analyzed as previously reported (Tamai & Ma, 2008).

Short-term experiment with Na⁺

We also conducted a short-term experiment with Na⁺. For this purpose, seedlings of both WT and *hak4* mutant lines were first grown in 1/2 strength solution until the flowering stage, followed by exposing to a solution containing 50 mM NaCl. After 2 days, different organs, including node I, flag leaf blade, flag leaf sheath, peduncle, and panicle were separated and subjected to Na⁺ determination as described below. Na⁺ distribution in each organ is calculated by (Na content in each organ/total Na content) × 100%.

Element mapping in the peduncle

The distribution pattern of Na⁺ in peduncle was observed by LA-ICP-MS. At the flowering stage, 4 g NaCl per pot was added to the soil and peduncle including the flag leaf sheath at ripening stage was sampled for analysis. The cross-sections of the peduncle containing flag leaf sheath of both wild-type rice and *hak4-1 + a* mutant were prepared as previously described with some modifications (Yamaji & Ma, 2019). Briefly, the following settings were used: energy, 1–2 J cm⁻² (approximately 2.5% of maximum energy); scan speed, 50 μm sec⁻¹; repetition rate, 20 Hz; laser spot size, 25 μm; and He carrier flow rate, 800 ml min⁻¹. All elemental signals were obtained with an Agilent Technologies 8900 ICP-MS device operated in helium mode. The time-course of ICP-MS output of the raster scanning was converted to element images using iQUANT2 software (Suzuki et al., 2018). Two biological replicates (from different plants) of each sample were made, showing similar results.

Mineral element concentration determination

All samples were dried at 70°C in an oven for 4 days. Dried samples were digested with concentrated HNO₃ (60%) at a temperature up to 140°C. The concentration of Na⁺ and K⁺ in the digested solution was determined by flame photometry (M410, Sherwood, UK) or ICP-MS (7700X; Agilent Technologies), while other mineral elements was determined by ICP-MS.

Statistical analysis

For electrophysiological results, the statistical analysis was performed using one-way ANOVA and Student's *t*-test with SPSS 20.0 software. For other results, Tukey's test and Duncan's test were applied to test differences among treatments at *P* < 0.05.

AUTHOR CONTRIBUTIONS

JC and JFM conceived and designed the experiments; JC performed most experiments; SFW and SY Y performed the transport activity assay; NY investigated the expression pattern in various organs at different growth stages; JC,

YX, NY, SFW, SY Y, YHS, RFS, and JFM analyzed data; JC and JFM wrote the manuscript; all authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Alignment and phylogenetic tree of HAK transporters.

Figure S2. pH-dependence of OsHAK4-mediated Na⁺ transport activity in oocyte.

Figure S3. Subcellular localization of OsHAK4.

Figure S4. Expression patterns of *OsHAK17*.

Figure S5. CRISPR/Cas9-induced mutations in the *OsHAK4* gene.

Figure S6. Phenotypic analysis of *OsHAK4* knockout lines at the vegetative growth stage.

Figure S7. Phenotypic analysis of *hak4* mutants at the reproductive growth stage.

Figure S8. Concentration of mineral elements in different organs of wild-type (WT) rice and *hak4* mutants at the flowering stage.

Table S1. Primer sequences used in this study.

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