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ORIGINAL ARTICLE

Full-length paper

Title:

**Expression level of the sodium transporter gene *OsHKT2;1* determines sodium accumulation of rice cultivars under potassium-deficient conditions**

Running title:

*OsHKT2;1* underlying the QTL for Na uptake

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**Abstract**

Under potassium (K)-deficient conditions, rice (*Oryza sativa* L.) actively takes up and utilizes sodium (Na) as an alternative element to K. In this study, we cloned a gene responsible for cultivar differences in shoot Na accumulation using a map-based cloning method. The responsible gene *OsHKT2;1* encodes an Na transporter associated with Na uptake in root tissues, and its expression level was positively correlated with Na uptake potential in 11 rice cultivars. We found that *OsHKT2;1* overexpression promoted shoot Na accumulation under low K supply and proposed that *OsHKT2;1* expression level is a key factor in the Na accumulation potential in rice cultivars. However, under sufficient K supply, *OsHKT2;1*-overexpressing rice plants accumulated Na in roots but not in shoots. This result suggests that Na transfer from root to shoot may be regulated by another Na transporter.

**Key words:** map-based cloning, *Oryza sativa*, *OsHKT2;1*, potassium, sodium.

## INTRODUCTION

Potassium (K) is an essential macronutrient for plants. Although K does not become a part of the chemical structure of plant tissue, it plays many important roles in growth. The roles of K in plants are summarized as follows: (1) activation of enzymatic reactions, (2) charge balancing, and (3) osmoregulation (Wakeel *et al.* 2011). Sodium (Na), which is an alkali metal like K, is not essential to plant growth. However, Na has beneficial effects on plant growth under K-deficient conditions (Flowers and Läuchli 1983; Takahashi *et al.* 1997; Takahashi and Maejima 1998). Although activation of enzymatic reactions is a K-specific function, Na substitutes for K in charge balancing and osmotic regulation to some extent (Takahashi and Maejima 1998; Wakeel *et al.* 2011).

Under low K supply, Na supplementation can improve the growth of rice (*Oryza sativa* L.) (Yoshida *et al.* 1969). Shoot Na concentrations in rice plants increased under low exchangeable soil K (Akai *et al.* 2012). Na uptake in K-deficient rice plants showed a positive correlation with grain filling ratio (Seino *et al.* 1992). We previously found that shoot Na accumulation under low K supply significantly differed among rice cultivars and that Na supplementation significantly improved the growth of the high Na accumulation cultivar Koshihikari but did not affect that of the low Na accumulation cultivar IR64 (Miyamoto *et al.* 2012). These results suggest that a high Na accumulation cultivar is beneficial for rice production by saving K fertilizer consumption.

In order to reveal what determines Na accumulation potential of rice cultivars, we need to identify a gene responsible for cultivar difference in Na accumulation under K-deficient conditions. According to quantitative trait loci (QTL) analysis using recombinant inbred lines (RILs) derived from a cross of Koshihikari and Kasalath, the major QTL associated with high Na accumulation in K-deficient rice plants was detected on chromosome 6, explaining 74% of the phenotypic variance (Miyamoto *et al.* 2012). The 6.4 Mbp

responsible chromosomal region contained a high-affinity $K^+$ Transport (HKT) transporter gene *OsHKT2;1*.

A HKT transporter was first isolated in wheat (*Triticum aestivum* L.) (Schachtman and Schroeder 1994) and characterized as a $K^+$-$Na^+$ cotransporter (Gassmann *et al*. 1996; Rubio *et al*. 1995). Seven functional *HKT* genes have been detected in the genome of the *japonica* rice cultivar Nipponbare (Huang *et al.* 2008). *OsHKT2;1* was isolated from Nipponbare (Horie *et al.* 2001), and characterized as a Na transporter using heterologous expression systems (Garciadeblás *et al.* 2003; Horie *et al.* 2001). Analysis using loss-of-function mutants confirmed that OsHKT2;1 was associated with Na uptake *in vivo* under low K supply (Horie *et al.* 2007).

In the present study, we performed map-based cloning of a gene responsible for cultivar difference in Na accumulation and revealed that the responsible gene was located in a 100 kbp chromosomal region containing *OsHKT2;1*. Here, we focused on expression levels of *OsHKT2;1* and found that its expression level was positively correlated with Na uptake in rice plants. Our results suggest that *OsHKT2;1* expression level is a key factor in the Na accumulation potential of rice cultivars.

## MATERIALS AND METHODS

### Plant materials

Seeds of CSSL 2031-15 were kindly provided by Dr. Yano from the National Institute of Agricultural Science (NIAS), Tsukuba, Japan. The genetic background of the line 2031-15 was an *indica* cultivar IR64 and its chromosome was partially substituted with a segment derived from a *japonica* cultivar Koshihikari. Genotyping with 148 markers covering 12 chromosomes revealed that the genotypes of the line 2031-15 were heterozygous at the positions of simple sequence repeat (SSR) markers (McCouch *et al.* 2002) RM1340,

RM5509, and RM5463. A subset of 74 lines showing a recombination event in the region between markers RM1340 and RM5463 were selected from a population produced by self-pollinations of the line 2031-15, and were used for map-based cloning of a gene responsible for cultivar difference. Although genotypes on parts of chromosome 1 and 11 had also been heterozygous in line 2031-15, they became homozygous for IR64 in some progeny lines. Therefore, we did not focus on the substituted regions on chromosomes 1 and 11 in this study.

Seeds of rice cultivars in the Japanese landrace core collection (JRC) (Ebana *et al.* 2008) and world rice collection (WRC) (Kojima *et al.* 2005) were obtained from NIAS. Hinode (JRC 3), Yamadabake (JRC 5), Okkamodoshi (JRC 8), Kahei (JRC 11), Oiran (JRC 12), Meguromochi (JRC 14), and Khau Tan Chiem (WRC 52) were used for this study.

*OsHKT2;1* overexpressing lines were produced by an *Agrobacterium*-mediated method (Toki *et al.* 2006). ORF of *OsHKT2;1* was amplified by PCR using PrimeSTAR® HS DNA Polymerase (TaKaRa Bio) and the *OsHKT2;1* ORF primers (Table 1). cDNA derived from Koshihikari was used as a template for PCR. The primer HKT2;1-F contained the sequence CACC for subsequent TOPO cloning. The amplified fragment was cloned into a Gateway pENTR/D-TOPO cloning vector (Invitrogen). The obtained plasmid was subcloned into pGWB502Ω (Nakagawa *et al.* 2007) containing 35S promoter using LR clonase (Invitrogen). The resulting vector was introduced into an *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.* 1993), which was used for transformation of rice plants (cv. Nipponbare).

**Growth conditions**

Pretreatments of rice seeds have previously been described in detail (Miyamoto *et al.* 2012). Seeds were incubated for three days at 30°**C** in distilled water with a fungicide, and

then sown on a nylon mesh framed with a plastic floater. The floater was placed onto 1 L of culture solution in a plastic container. Plants were cultured in a growth chamber with a photoperiod of 12-h light/12-h dark at 350 μmol $m^{-2}$ $s^{-1}$ of photo intensity at 30°**C** under 80% relative humidity.

A hydroponic culture solution was prepared with distilled water. A solution of control treatment composed of 0.75 mol $m^{-3}$ $(NH_4)SO_4$, 0.25 mol $m^{-3}$ $(NH_4)_2HPO_4$, 0.75 mol $m^{-3}$ KCl, 0.50 mol $m^{-3}$ $CaCl_2$, 0.50 mol $m^{-3}$ $MgCl_2$, 0.03 mg $m^{-3}$ Fe-citrate (Nacalai Tesque), and micronutrients (Hewitt 1966). Potassium chloride (KCl) supply was reduced for low K treatment, and sodium chloride (NaCl) was added for Na supplementation. The solution pH was adjusted to 6.0 with hydrochloric acid (HCl).

**Map-based cloning of a responsible gene**

Ten plants of Koshihikari, IR64, and four CSSLs were grown for 10 days in a solution containing 0.08 mol $m^{-3}$ KCl and 0.38 mol $m^{-3}$ NaCl. They were individually harvested and separated into shoots and roots. Shoots were subjected to analysis of Na concentration and roots were used for DNA extraction with cetyl trimethyl ammonium bromide (CTAB) buffer. Extracted DNA was used as a template for polymerase chain reaction (PCR) performed with Brend Taq (TOYOBO). Fourteen SSR markers and the marker 1700-1900 (Table 1) were used for genotyping.

**Expression analysis of candidate responsible genes**

Thirty plants of Koshihikari or IR64 were grown for seven days in a solution containing 0.75 mol $m^{-3}$ KCl and then transferred into a solution containing 0.75 (+ K) or 0 (− K) mol $m^{-3}$ KCl. EVéry solution was supplemented with 0.38 mol $m^{-3}$ NaCl. Four days later, the

plants were divided into three groups and their shoots and roots were separated. These samples were used for RNA extraction.

Expression level of *OsHKT2;1* in Koshihikari, IR64 and CSSL 2031-15-87-71, which has a Koshihikari allele in the responsible chromosomal region, was analyzed. Fifteen plants of each cultivar or line were grown for seven days in a solution containing 0.38 mol $m^{-3}$ KCl and 0.38 mol $m^{-3}$ NaCl. The plants were divided into three groups and their roots were used for the analysis.

**Analysis of relationship between *OsHKT2;1* expression level and Na uptake**

Two *temperate japonica* cultivars, Koshihikari and Sasanishiki; seven tropical *japonica* cultivars, Hinode, Yamadabake, Okkamodoshi, Kahei, Oiran, Meguromochi, and Khau Tan Chiem; and two *indica* cultivars, IR64 and Kasalath, were used for the analysis. Plants were grown in a solution containing 0.38 mol $m^{-3}$ potassium chloride (KCl) with 0.38 mol $m^{-3}$ sodium chloride (NaCl) supplement. Koshihikari and the other five cultivars were cultured in a container, and values in the two containers were normalized to those of Koshihikari. For analysis of Na concentration, 10 plants were grown for 10 days and divided into three groups. Whole plant samples (shoot + root) were used for the analysis. For analysis of the expression level *OsHKT2;1*, 15 plants were grown for seven days and divided into three groups. Their roots were used for the analysis.

**Evaluation of Na accumulation potential in *OsHKT2;1*-overexpressing lines**

Wild-type and *OsHKT2;1*-overexpressing lines were grown in a solution containing 0.75 (control) or 0.08 (low K) mol $m^{-3}$ KCl with 0.38 mol $m^{-3}$ NaCl supplementation. For analysis of *OsHKT2;1* expression level, 15 plants were grown for ten days and divided into three groups. Whole plant samples (shoots + roots) were used for the analysis. For analysis

of K and Na concentrations, ten plants were grown for ten days and divided into three groups. Their shoots and roots were separately used for the analysis.

**Measurement of K and Na concentration**

Samples were prepared as previously described (Miyamoto *et al.* 2012). The sample was digested with $HNO_3$–$H_2SO_4$ and the digested solution was diluted with 100 mol $m^{-3}$ HCl. K and Na concentrations in the solution were determined by flame photometry.

**Quantitative gene expression analyses**

Shoot or root samples were ground in liquid nitrogen. Total RNA was extracted from powdered samples with the RNeasy Plant Mini Kit (Qiagen) followed by digestion with Recombinant DNaseI (TaKaRa Bio). RNA concentration in the solution was determined by spectrometry (Bio Spec-mini, Shimadzu Seisakusyo, Kyoto, Japan). First-strand cDNA was synthesized from 2 μg of total RNA using the ReverTra Ace® (TOYOBO) and oligo $(dT)_{20}$ primer. Synthesized cDNAs were used for PCR amplification. Quantitative real-time PCR (qRT-PCR) was performed with the thermal cycler dice real time system (Takara Bio) using the THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO). Expression levels of *Ubiquitin* and *Actin 1* were used as internal controls.

**Primer sequences**

The sequences of primers used in this study are listed in Table 1.

**RESULTS**

**Fine mapping of a responsible gene**

Using CSSLs derived from Koshihikari and IR64, fine mapping of a gene responsible for cultivar difference in shoot Na accumulation was performed (Fig. 1). The genetic background of CSSL 2031-15 is IR64, and the terminal region from the marker RM1340 to RM5463 on chromosome 6 is heterozygous (Fig. 1a). CSSLs 2031-15-123-3-32 (123-3-32), 2031-15-35-9-368 (35-9-368), 2031-15-123-3-63 (123-3-63), 2031-15-123-3-22 (123-3-22), 2031-15-35-9-135 (35-9-135), 2031-15-87-81-2 (87-81-2), 2031-15-123-3-56 (123-3-56), and 2031-15-35-9-374 (35-9-374) were produced by self-pollinations of the line 2031-15. Their chromosomes had recombination events in the region between markers RM1340 and RM5463 (Fig. 1a). In parent cultivars and progeny of the line 2031-15, shoot Na accumulation was significantly higher in Koshihikari or Koshihikari type progeny compared with that in IR64 or IR64 type progeny (Fig. 1b). Shoot Na accumulation was higher in all progeny of 123-3-32, 35-9-368 and 123-3-63 than in the parent cultivar IR64. In contrast, shoot Na accumulation was low in all progeny of 123-3-56 or 35-9-374, as in IR64. In lines 123-3-22, 35-9-22, and 87-81-2, shoot Na accumulation was higher in Koshihikari-type compared with that in IR64-type progeny. The progeny test revealed that a gene responsible for cultivar difference in shoot Na accumulation was located in a 150-kbp region between markers RM20657 and 1700-1900 (Fig. 1a).

**Expression response of candidate genes to potassium deprivation**

According to the Rice Annotation Project Database (RAP-DB) (Rice Annotation Project 2008), 21 genes are located in the candidate region. We evaluated expression changes of the 21 genes under K deprivation (Table 2). In Koshihikari, the expression levels of *Os06g0699850* and *Os06g0701700* significantly increased, and those of *Os06g0699600, Os06g0700100*, *Os06g0700500* and *Os06g0700700* significantly decreased. In contrast, in IR64, the expression levels of *Os06g0700601, Os06g0701100*, and *Os06g0701700*

significantly increased. Among these genes, *Os06g0701700* encodes an Na transporter, OsHKT2;1, which is associated with Na uptake in K-starved roots (Horie *et al.* 2007). K deprivation increased the expression level of *OsHKT2;1* by more than 2-fold in both Koshihikari and IR64. The expression level of *OsHKT2;1* was significantly higher in Koshihikari compared with that in IR64. This result suggested that *OsHKT2;1* is a gene responsible for cultivar differences in shoot Na accumulation. The candidate region contained another HKT-type transporter gene *Os06g0701600. Os06g0701600* encodes a K-selective transporter OsHKT2;4 (Horie *et al.* 2011; Lan *et al.* 2010; Sassi *et al.* 2012), and its expression level did not significantly change under K deprivation. Thus *OsHKT2;4* is unlikely to be associated with Na accumulation under low K.

The expression levels of *OsHKT2;1* in Koshihikari, IR64 and CSSL 2031-15-87-71 (87-71), which has a Koshihikari-type allele in the candidate chromosomal region, were evaluated (Fig. 2). Although the expression level of *OsHKT2;1* was significantly lower in 87-71 compared with that in Koshihikari, it was 2-fold higher in 87-71 compared with that in IR64. In addition, we found that *OsHKT2;1* expression was enhanced also in another line having a Koshihikari-type allele in the candidate region (data not shown). This result showed that a Koshihikari-type allele in *OsHKT2;1* could enhance *OsHKT2;1* expression.

**Relationship between *OsHKT2;1* expression level and sodium uptake potential in various rice cultivars**

The relationships between *OsHKT2;1* expression levels and Na uptake potential in Koshihikari, Sasanishiki, Hinode, Yamadabake, Okkamodoshi, Kahei, Oiran, Meguromochi, Khau Tan Chiem, IR64, and Kasalath were investigated (Fig. 3). Pearson's correlation coefficient (0.697) indicated that Na uptake had a significant positive correlation with *OsHKT2;1* expression in roots in these 11 cultivars ($P < 0.05$). In

Koshihikari, Sasanishiki, and Meguromochi, both *OsHKT2;1* expression levels in roots and Na concentrations in whole plants were relatively high. In contrast, either *OsHKT2;1* expression or Na uptake was low in Hinode, Yamadabake, Okkamodoshi, Kahei, Oiran, IR64, and Kasalath. Although Khau Tan Chiem, like Koshihikari, exhibited high Na uptake, its expression level of *OsHKT2;1* was as low as that of IR64.

**Effect of enhanced *OsHKT2;1* expression on sodium accumulation**

Using *OsHKT2;1*-overexpressing lines (2-9, 4-2), the effect of enhanced *OsHKT2;1* expression on Na accumulation was evaluated. The expression level of *OsHKT2;1* was 200- or 18-fold higher in lines 2-9 or 4-2 compared with that in the wild type (Fig. 4). In the wild type, 2-9, and 4-2, shoot dry weight did not significantly differ between treatments, and it was lower in lines 2-9 and 4-2 compared with in the wild type (Fig. 5a). Shoot dry weight of the wild type was decreased by more than 40% under the low-K treatment without Na supplementation compared to the control treatment (data not shown). Therefore, the shoot growth of the wild type, 2-9, and 4-2 in the low-K treatment would be improved by Na supplementation (Fig. 5a). Root dry weight in the control treatment was significantly lower in lines 2-9 and 4-2 compared with that in the wild type (Fig. 5b). The root growth of the wild type decreased under the low-K treatment but those of 2-9 and 4-2 did not (Fig. 5b). Shoot K concentration was slightly higher in lines 2-9 and 4-2 compared with that in the wild type in the control treatment (Fig. 5c) because shoot dry weight was lower in 2-9 and 4-2 compare with that in the wild type (Fig. 5a). Root K concentration under the control treatment was significantly lower in 2-9 compared with that in the wild type (Fig 5d). Although shoot Na concentration in the control treatment was similarly low in the wild type, 2-9, and 4-2, it was significantly higher in lines 2-9 and 4-2 compared with that in the wild type under the low-K treatment (Fig. 5e). Root Na concentration was

significantly higher in lines 2-9 and 4-2 compared with that in the wild type in the control treatment (Fig. 5f). Root Na concentration in the low-K treatment was slightly higher in 2-9 and not in 4-2 compared with that in the wild type (Fig. 5f). It may be because Na translocation from root to shoot was promoted under the low-K treatment. This analysis revealed that enhanced expression levels of *OsHKT2;1* conferred high Na uptake potential on K-deficient rice plants.

## DISCUSSION

### *OsHKT2;1* is a gene responsible for cultivar differences in sodium uptake under potassium-deficient conditions

The map-based cloning of a gene responsible for cultivar differences in shoot Na accumulation revealed that a responsible gene was located on the terminal end of chromosome 6 (Fig. 1). This chromosomal region contains two cation transporter genes, *OsHKT2;1* and *OsHKT2;4* (Table 2). The expression level of *OsHKT2;4* was much lower in roots compared with that in shoots and did not change under K deprivation (Table 2). Horie *et al.* (2011) and Lan *et al.* (2010) reported that a cation transporter encoded by *OsHKT2;4* exhibited permeability to a broad range of monovalent or divalent cations, although it was strongly permeable to $K^+$. Sassi *et al.* (2012) reported that OsHKT2;4 was a $K^+$-selective transporter and could possibly mediate $K^+$-$Na^+$ symport when the external concentration of $K^+$ was $10^2$ or $10^3$ times lower than that of $Na^+$. Considering these previous studies, *OsHKT2;4* is unlikely to be associated with shoot Na accumulation under low K supply. On the other hand, OsHKT2;1 was characterized as an Na-selective transporter using heterologous expression systems (Garciadeblás *et al.* 2003; Horie *et al.* 2001). Golldack *et al.* (2002) showed that OsHKT2;1 could restore growth in a K uptake-defective yeast strain. However, Horie *et al.* (2007) reported that loss of OsHKT2;1

function by *Tos17* insertion diminished rice Na uptake *in vivo* but did not affect Rb (K tracer) uptake. Microarray analysis showed that *OsHKT2;1* expression was upregulated by more than 2-fold in root tissues within 6 h after K deprivation (Ma *et al.* 2012). Using a heterologous expression system Oomen *et al.* (2012) reported that Na transport activity of the OsHKT2;1 protein was highly conserved in 49 rice cultivars. In the present study we showed that expression of *OsHKT2;1* was higher in the CSSL having a Koshihikari-type allele in the responsible region compared with that in IR64 (Fig. 2). In addition, the expression level of *OsHKT2;1* was positively correlated with Na uptake potential in various rice cultivars (Fig. 3), and an enhanced *OsHKT2;1* expression level promoted shoot Na accumulation under low K supply (Fig. 5). It may be inferred that the *OsHKT2;1* expression level determines the Na uptake potential in rice cultivars under K-deficient conditions.

A Koshihikari-type allele in the responsible chromosomal region enhanced Na expression level of *OsHKT2;1* (Fig. 2). This finding suggests that the *OsHKT2;1* promoter was activated in a Koshihikari-type allele. DNA polymorphisms in a promoter region may induce phenotypic variation in wheat (Nakamura *et al.* 2011) or soybean cultivars (Jiang *et al.* 2013) by changing gene expression levels. Horie *et al.* (2007) confirmed that a 1.6-kbp upstream sequence could regulate *OsHKT2;1* expression, depending on external K levels. Differences in promoter activity between Koshihikari and IR64 may affect the *OsHKT2;1* expression level. Using reporter genes, we need to compare the activity of the *OsHKT2;1* promoter between Koshihikari and IR64.

**Multiple sodium transport mechanisms are involved in potassium substitution by sodium in rice plants**

Although shoot Na accumulation was promoted in CSSLs having a Koshihikari-type allele in the responsible chromosomal region, it did not attain that of Koshihikari (Fig. 1). One of the possible reasons is that *OsHKT2;1* expression remained lower in CSSL compared with that in Koshihikari (Fig. 2). In addition, shoot Na accumulation in K-deficient rice plants may rely on various Na transport mechanisms, such as uptake in root tissues, translocation from root to shoot, and distribution in shoot tissues. In the analysis of the relationship between *OsHKT2;1* expression and Na uptake potential, Khau Tan Chiem exhibited high Na uptake, although its *OsHKT2;1* expression level was relatively low (Fig. 3). This suggests that the other Na transporters accounted for the high Na uptake in Khau Tan Chiem. *OsHKT2;1*-overexpressing rice plants accumulated Na in roots but not in shoots under sufficient K supply (Fig. 5e, f). *In situ* hybridization confirmed that *OsHKT2;1* was highly expressed in the epidermis, exodermis, cortex (Jabnoune *et al.* 2009), and vascular tissues (Golldack *et al.* 2002) in K-starved roots. β-glucuronidase (GUS) and green fluorescence protein (GFP) reporter assays revealed that *OsHKT2;1* was expressed not only in the cortex but also in the endodermis in K-starved roots (Horie *et al.* 2007). These reports suggest that *OsHKT2;1* may be involved not only in Na uptake into roots but also in Na loading into xylem. However, our finding indicated that Na uptake in roots could be activated by OsHKT2;1 but that Na transfer from root to shoot was also regulated by other genes. Previously, *OsHKT1;5* has been cloned as the quantitative trait locus (QTL) *SKC1* associated with salt tolerance of an *indica* cultivar Nona Bokra and it suppressed Na accumulation in shoots via retrieval of Na from xylem (Ren *et al.* 2005). Arabodopsis HKT1;1 controlled shoot $Na^+$ accumulation by withdrawal of $Na^+$ from xylem in root (Davenport *et al*. 2007), which is similar to the function of OsHKT1;5. Berthomieu *et al*. (2003) reported that AtHKT1;1 associated with $Na^+$ recirculation from shoot to root via phloem. OsHKT1;5 also may control shoot $Na^+$ accumulation via xylem or phloem. A rice

salt overly sensitive 1 (SOS1) encodes the $Na^+$/ $H^+$ exchanger, which is the functional homolog of Arabidopsis SOS1 and conferred salt tolerance to rice plants (Martínez-Atienza *et al*. 2007). AtSOS1 was localized in plasma membrane and preferentially expressed in xylem parenchyma cells of roots, hypocotyls, inflorescence stems, and leaves (Shi *et al*. 2002). Shoot Na concentration was lower in the *sos1* mutant under mild salt stress (25 mM NaCl) but higher in the mutant under severe salt stress (100 mM NaCl) compared to that in the wild type (Shi *et al*. 2002). It suggests that SOS1 protein controls $Na^+$ translocation from root to shoot. The mechanism of shoot $Na^+$ accumulation regulated by these transporter proteins should be investigated.

In our previous study, high Na accumulation potential improved growth under low K supply (Miyamoto *et al.* 2012). In the present study, shoot Na accumulation under low K supply was significantly higher in *OsHKT2;1*-overexpressing lines (Fig. 5e), but shoot growth did not differ between the wild type and lines 2-9 or 4-2 under the low-K treatment (Fig. 5a). Not only shoot Na accumulation but also Na distribution in shoot tissues is important for the K substitution effect by Na in rice plants. Recently, *OsHKT1:4* has been reported to control $Na^+$ transfer from sheath to blade in rice shoots (Cotsaftis *et al.* 2012). *OsHKT1;1* and *OsHKT1;3* encodes $Na^+$-selective transporters (Jabnoune *et al*. 2009). The former was expressed mainly in root epidermis, exodermis, and cortex though the expression level was lower than that of *OsHKT2;1* (Jabnoune *et al*. 2009). The latter was preferentially expressed in mature leaves and root phloem (Jabnoune *et al*. 2009). They may control $Na^+$ distribution *in planta*. Fukuda *et al.* (2004) showed that OsNHX1 was $Na^+$/ $H^+$ antiporter working on $Na^+$ compartmentation into vacuole. They suggested that OsNHX1 was important for suppression of $Na^+$ transfer from old leaves to young ones. Various Na transporters are involved in Na distribution in rice, and their association with the K substitution effect by Na should be investigated.

## CONCLUSION

In this study, we showed that the QTL gene *OsHKT2;1* affected the Na uptake potential in rice cultivars via its expression level. In addition, our study suggested that Na transfer from root to shoot is regulated by another Na transporter. For breeding a new rice cultivar tolerating low K input, we need to identify the other genes associated with K substitution by Na in rice plants.

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**Figure legends**

**Figure 1** Fine mapping of a gene responsible for cultivar difference in shoot sodium accumulation. (A) A responsible gene was mapped onto a 150-kbp region between markers RM20657 and 1700-1900 using 1480 progeny of chromosome segment substitution lines (CSSLs) derived from Koshihikari and IR64. A black-filled or open region indicates a Koshihikari or IR64 type chromosome. The number of recombinant lines is shown between the markers. (B) Shoot sodium (Na) contents of Koshihikari, IR64 and progeny of CSSLs were shown. Black-filled bars indicate values of Koshihikari or Koshihikari type progenies (Ko), and open bars indicate values of IR64 and IR64 type progenies (IR). Plants were grown in 1 L of a solution containing 0.08 mol $m^{-3}$ potassium chloride (KCl) and 0.38 mol $m^{-3}$ sodium chloride (NaCl). Ten-day-old seedlings were harvested. Values are means with standard deviations (SD). Replicates were as followings; Koshihikari (n = 20), IR64 (n = 19), 2031-15 (Ko, n = 16; IR, n = 4), 2031-15-123-3-32 (n = 16), 2031-15-35-9-368 (Ko, n = 6; IR, n = 5), 2031-15-123-3-63 (Ko, n = 3; IR, n = 7), 2031-15-123-3-22 (Ko, n = 6; IR, n = 4), 2031-15-35-9-135 (Ko, n = 4; IR, n = 7), 2031-15-87-81-2 (Ko, n = 4; IR, n = 6), 2031-15-123-3-56 (Ko, n = 7; IR, n = 4) and 2031-15-35-9-374 (n = 19). The means of parent cultivars were used for correction of values among cultivations. Significant difference between Koshihikari and IR64 or Koshihikari type and IR64 type progenies is indicated by an asterisk (**$P < 0.01$, Student's t-test).

**Figure 2** *OsHKT2;1* relative expression in a chromosome segment substitution line. The line 2031-15-87-71 (87-71) has a Koshihikari-type allele in the candidate chromosomal region. Koshihikari, IR64, and 87-71 were grown for seven days in a solution containing 0.38 mol $m^{-3}$ potassium chloride (KCl) with 0.38 mol $m^{-3}$ sodium chloride (NaCl) supplement. *OsHKT2;1* expression levels in roots were evaluated by quantitative Real-time PCR (qPT-PCR). Expression levels of *Ubiquitin* and *Actin 1* were used as internal controls. Values are means with standard deviations (SD) (n = 3). Variance among cultivars was significant by analysis of variance (ANOVA) ($P < 0.01$).

**Figure 3** Relationship between *OsHKT2;1* expression and sodium uptake. Eleven rice cultivars including Koshihikari, Sasanishiki, Hinode, Yamadabake, Okkamodoshi, Kahei, Oiran, Meguromochi, Khau Tan Chiem, IR64, and Kasalath were used for the experiment. Plants were grown in a solution containing 0.38 mol $m^{-3}$ potassium chloride (KCl) with

0.38 mol $m^{-3}$ sodium chloride (NaCl) supplement. Whole plants of ten-day-old seedlings were used for analysis of Na concentration, and roots of seven-day-old plants were used for analysis of *OsHKT2;1* expression level. Expression levels of *Ubiquitin* and *Actin 1* were used as internal controls for gene expression analysis. Values are means with standard deviations (SD) (n = 3). By Pearson's correlation coefficient (0.697), the correlation between Na concentration and *OsHKT2;1* relative expression level was significant ($P < 0.05$).

**Figure 4** Overexpression of *OsHKT2;1* in transgenic lines. Wild-type (cv. Nipponbare) and *OsHKT2;1*-overexpressing lines (2-9, 4-2) were grown for ten days in a solution containing 0.75 (control) or 0.08 (low K) mol $m^{-3}$ potassium chloride (KCl) with 0.38 mol $m^{-3}$ sodium chloride (NaCl) supplement. Expression of *OsHKT2;1* in whole plants was evaluated by quantitative Real-time PCR (qPT-PCR). The expression levels of *Ubiquitin* and *Actin 1* were used as internal controls. Values are means with standard deviations (n = 3). Significant variance was detected between treatments or lines, and their interaction was also significant [$P < 0.01$, two-way analysis of variance (ANOVA)].

**Figure 5** Sodium accumulation potential in *OsHKT2;1*-overexpressing lines. Shoot or root dry weight (A, B), potassium (K) concentration (C, D) and sodium (Na) concentration (E, F) of wild type (cv. Nipponbare) and transgenic lines overexpressing *OsHKT2;1* (2-9, 4-2) are shown. Plants were grown for ten days in a solution containing 0.75 (control) or 0.08 (low K) mol $m^{-3}$ potassium chloride (KCl) with 0.38 mol $m^{-3}$ sodium chloride (NaCl) supplement. Values are means with standard deviation (SD) (n = 3). Significance of variance in each factor was as followings; shoot dry weight: treatment$^{n.\ s.}$, line$^{**}$, interaction$^{n.\ s.}$; root dry weight: treatment$^{**}$, line$^{**}$, interaction$^{**}$; shoot Na concentration: treatment$^{**}$, line$^{*}$, interaction$^{*}$; root Na concentration: treatment$^{**}$, line$^{**}$, interaction$^{**}$; shoot K concentration: treatment$^{**}$, line$^{*}$, interaction$^{**}$; root K concentration: treatment$^{**}$, line$^{**}$, interaction$^{**}$ [n. s., not significant; **, $P < 0.01$; *, $P < 0.05$, two-way analysis of variance (ANOVA)].

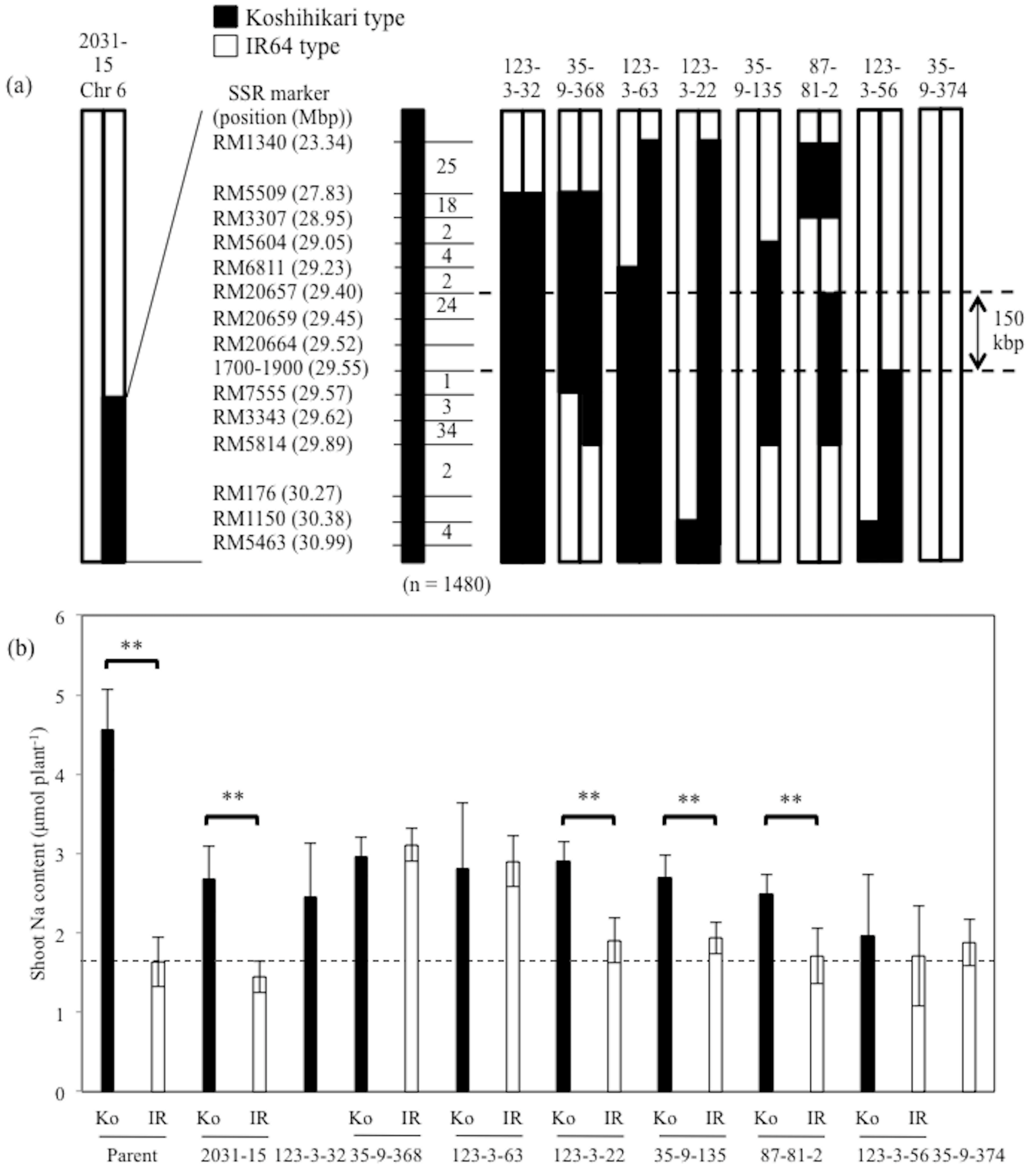
**Table 1** Primer sequences used for genotyping, quantitative gene expression analysis, or transformation in this study

| | Primer sequences | |
|---|---|---|
| | forward | reverse |
| For genotyping | | |
| 1700-1900 | CCTTCCTAGGGCTAGGGGTA | TGGACCTAGATAGGCCACAAA |
| For qRT-PCR | | |
| Os06g0699400 | TCATCTCAACCTCTTTCTAATGACC | CAGATCGCAGTTTGCATTAACC |
| Os06g0699500 | ACATCATCAAGGACTGCTCCAA | GCCGCCAATGGACATGA |
| Os06g0699600 | GGCTGCGATGCACTTGA | GCTTCCTTCCCGGATAACAC |
| Os06g0699700 | CTGGAAGCAAAGCCAATCAAA | GGAGGTCAACAATCATCAAGTTCTC |
| Os06g0699800 | TCCCAAATCCATGCATAATCAC | CCTTGCTGCCTCCTTGATG |
| Os06g0699850 | GCGACGGCAATCGAGTTA | TTCTCCTCCTCTTCTTCCTCCTC |
| Os06g0699900 | GCAGGTAGACCCAAGACAATCAC | CTTAGGATCACAAAACCCTCAAGAA |
| Os06g0700000 | AGCTACAGAGGTGGAGTTGAAGGA | CCTTCGTAGGTGGAGTGAGTATGAA |
| Os06g0700100 | GAATGGAAAGCCAGCAGAGG | CACGCTGAGATGACTGCAAGA |
| Os06g0700300 | AGGTACGTGCTGTGCTCGTC | GGACTCGGACTGGAAGATGG |
| Os06g0700500 | CATGTTCTTGACACCCAGTTCATT | CCATTAAATATCGGACTAGCGTGAA |
| Os06g0700550 | GCTTCTTGGTGTCGTGCAG | GCAAAGAGCCAAGCAACCA |
| Os06g0700601 | CCTTTTGTGGTGCCATGCT | CTGGGTTCATCGTCTTCCTCTT |
| Os06g0700700 | GTGATCGTTCCCTCCAGGAC | TTCCCATAAGCCCGAACC |
| Os06g0700750 | CTCGAGCGTGAGAGGAGAGATA | CTCCACCTTTTCCGCTCTCT |
| Os06g0701100 | AGTCCCTCCGTCCTGATTACA | GCATTGTAGCAGAGAACACACCA |
| Os06g0701200 | TTGTAGCTGGTGGGCTTGGT | AATTTGCAGCTCGCCTCCT |
| Os06g0701300 | TTAGCAAGGAACCTCCACACAA | CATCTCCGCAAAGGCTGAC |
| Os06g0701400 | AGTCCTCCTTCTCCATGGTCTC | AGCAGCACCTCCACCAATC |
| Os06g0701600 | TCGATCTCCACTGACCCTCTC | GGCAAGCAATCCCATCCT |
| Os06g0701700 | ATGGCAGTGAACGCAAGG | GTGCAAATGTTGTCGATGGTG |
| Ubiquitin | AGAAGGAGTCCACCCTCCACC | GCATCCAGCACAGTAAAACACG |
| Actin 1 | ATCCTTGTATGCTAGCGGTCGA | ATCCAACCGGAGGATAGCATG |
| For transformation | | |
| OsHKT2;1 ORF | CACCATGACGAGCATTTACCATGATT | TTACCATAGCCTCCAATATTCAC |

**Table 2** Expression change of candidate responsible genes under potassium deprivation Seven-day-old seedlings were transferred into 0.75 (+ K) or 0 (− K) mol $m^{-3}$ potassium chloride (KCl), and four days later shoots and roots were harvested. Expression levels of candidate responsible genes were evaluated by quantitative real-time PCR. The expression levels of *Ubiquitin* and *Actin 1* were used as internal controls. Values relative to shoot values under the + K treatment are shown ($n$ = 3). Difference was significant between values followed by different letters within a gene [$P < 0.05$, Tukey's honestly significant difference (HSD) test].

| | | | Relative expression level | | | |
|---|---|---|---|---|---|---|
| | | | Shoot | | Root | |
| Gene | Annotation | Cultivar | + K | − K | + K | − K |
| Os06g0699400 | MAP kinase 2 | Koshihikari | 1.00 ac | 1.23 c | 0.525 ab | 0.635 ab |
| | | IR64 | 0.668 ab | 0.470 b | 0.473 b | 0.244 b |
| Os06g0699500 | Tautomerase domain containing protein | Koshihikari | 1.00 a | 0.335 a | 0.0140 a | 0.0803 a |
| | | IR64 | 0.531 a | 1.01 a | 0.0944 a | 0.115 a |
| Os06g0699600 | CCT domain containing protein | Koshihikari | 1.00 ab | 0.558 ab | 1.93 b | 0.130 a |
| | | IR64 | 0.189 a | 0.163 a | 0.169 a | 0.131 a |
| Os06g0699700 | Similar to Aminodeoxychorismate synthase/ glutamine amidotransferase | Koshihikari | 1.00 a | 0.682 ab | 0.156 c | 0.111 c |
| | | IR64 | 0.499 b | 0.402 bc | 0.155 c | 0.104 c |
| Os06g0699800 | ENTH/ VHS domain containing protein | Koshihikari | 1.00 a | 1.10 a | 0.114 bc | 0.0683 c |
| | | IR64 | 1.09 a | 0.882 ab | 0.0710 c | 0.127 bc |
| Os06g0699850 | Hypothetical protein | Koshihikari | 1.00 a | 5.03 b | 0.220 a | 1.13 a |
| | | IR64 | 0.593 a | 0.734 a | 0.264 a | 0.301 a |
| Os06g0699900 | Proteasome/ cyclosome, regulatory subunit domain containing protein | Koshihikari | 1.00 a | 0.993 a | 0.595 bcd | 0.552 bcd |
| | | IR64 | 0.728 ab | 0.814 ad | 0.409 c | 0.432 bc |
| Os06g0700000 | Peptidase S8, subtilisin-related domain containing protein | Koshihikari | 1.00 a | 1.17 a | 0.378 bc | 0.204 bc |
| | | IR64 | 0.412 bc | 0.520 b | 0.0323 c | 0.248 bc |
| Os06g0700100 | Pentatricopeptide repeat domain containing protein | Koshihikari | 1.00 abc | 1.22 ab | 1.45 b | 0.864 ac |
| | | IR64 | 0.876 ac | 0.792 ac | 0.580 c | 0.694 c |
| Os06g0700300 | Conserved hypothetical protein | Koshihikari | 1.00 a | 1.79 a | 1.14 a | 1.96 a |
| | | IR64 | 2.60 a | 2.75 a | 2.48 a | 1.44 a |
| Os06g0700500 | Protein of unknown function DUF266, plant family protein | Koshihikari | 1.00 a | 0.946 a | 0.850 a | 0.377 b |
| | | IR64 | 1.07 a | 0.864 a | 0.823 a | 1.14 a |
| Os06g0700550 | Hypothetical protein | Koshihikari | 1.00 a | 1.06 a | $3.01 \times 10^3$ b | $2.59 \times 10^3$ b |
| | | IR64 | 0.424 a | 0.627 a | $3.04 \times 10^3$ b | $2.56 \times 10^3$ b |
| Os06g0700601 | Hypothetical gene | Koshihikari | 1.00 a | 1.06 a | 5.09 b | 3.17 ab |
| | | IR64 | 0.726 a | 1.04 a | 3.86 b | 4.15 a |

| | | | | | | |
|---|---|---|---|---|---|---|
| Os06g0700700 | Similar to P1B-type heavy metal transporting ATPase | Koshihikari | 1.00 a | 0.893 a | 7.45 b | 2.77 a |
| | | IR64 | 0.838 a | 0.936 a | 3.11 a | 3.04 a |
| Os06g0700750 | Conserved hypothetical protein | Koshihikari | 1.00 ab | 1.44 a | 0.010 b | 0.007 b |
| | | IR64 | 1.45 a | 1.25 ab | 0.000467 b | 0.000627 b |
| Os06g0701100 | Eukaryotic initiation factor 4A (elF4A) (elF-4A) | Koshihikari | 1.00 ac | 1.03 ac | 1.01 ac | 0.334 c |
| | | IR64 | 1.05 a | 1.87 b | 0.545 ac | 0.683 ac |
| Os06g0701200 | UTP-glucose-1-phosphate uridylyltransferase family protein | Koshihikari | 1.00 a | 1.08 a | 0.685 c | 0.485 cd |
| | | IR64 | 0.211 bd | 0.135 b | 0.134 b | 0.0898 b |
| Os06g0701300 | Similar to ABC1 family protein | Koshihikari | 1.00 a | 1.08 a | 0.322 b | 0.453 b |
| | | IR64 | 0.502 b | 0.601 b | 0.294 b | 0.297 b |
| Os06g0701400 | Similar to 60S acidic ribosomal protein P3 (P1/ P2-like) (P3A) | Koshihikari | 1.00 a | 0.479 a | 0.678 a | 0.337 a |
| | | IR64 | 1.28 a | 0.597 a | 0.414 a | 0.271 a |
| Os06g0701600 | OsHKT2;4 | Koshihikari | 1.00 a | 1.00 a | 0.000199 c | 0.00109 c |
| | | IR64 | 1.97 a | 1.47 ab | 0.000256 c | 0.000103 c |
| Os06g0701700 | OsHKT2;1 | Koshihikari | 1.00 a | 8.10 ac | 24.7 d | 70.3 e |
| | | IR64 | 2.25 ab | 4.51 ac | 16.6 bcd | 42.5 f |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IR64 type
2031-15 Chr 6
SSR marker (position (Mbp))
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RM5509 (27.83)
RM3307 (28.95)
RM5604 (29.05)
RM6811 (29.23)
RM20657 (29.40)
RM20659 (29.45)
RM20664 (29.52)
1700-1900 (29.55)
RM7555 (29.57)
RM3343 (29.62)
RM5814 (29.89)
RM176 (30.27)
RM1150 (30.38)
RM5463 (30.99)
25
18
2
4
2
24
1
3
34
2
4
(n = 1480)
123-3-32
35-9-368
123-3-63
123-3-22
35-9-135
87-81-2
123-3-56
35-9-374
150 kbp
(b)
Shoot Na content (μmol plant-1)
**
Ko
IR
Parent
2031-15
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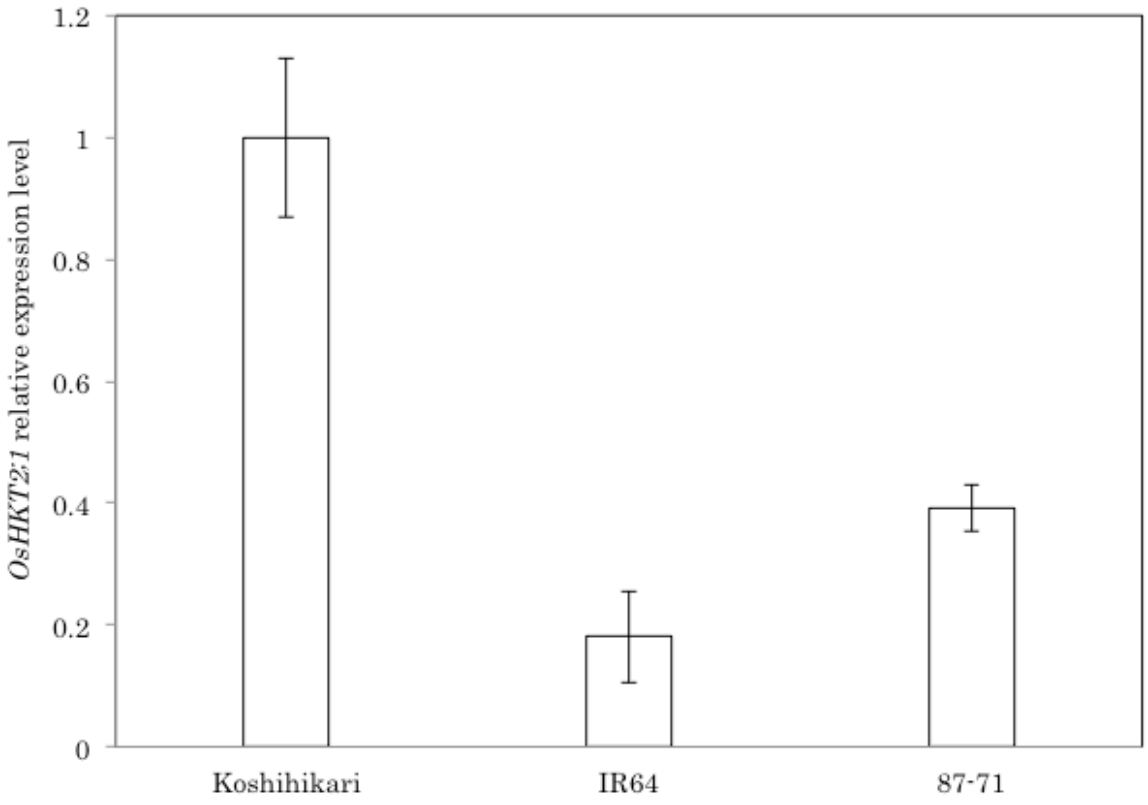
35-9-135
87-81-2
123-3-56
35-9-374

Fig. 1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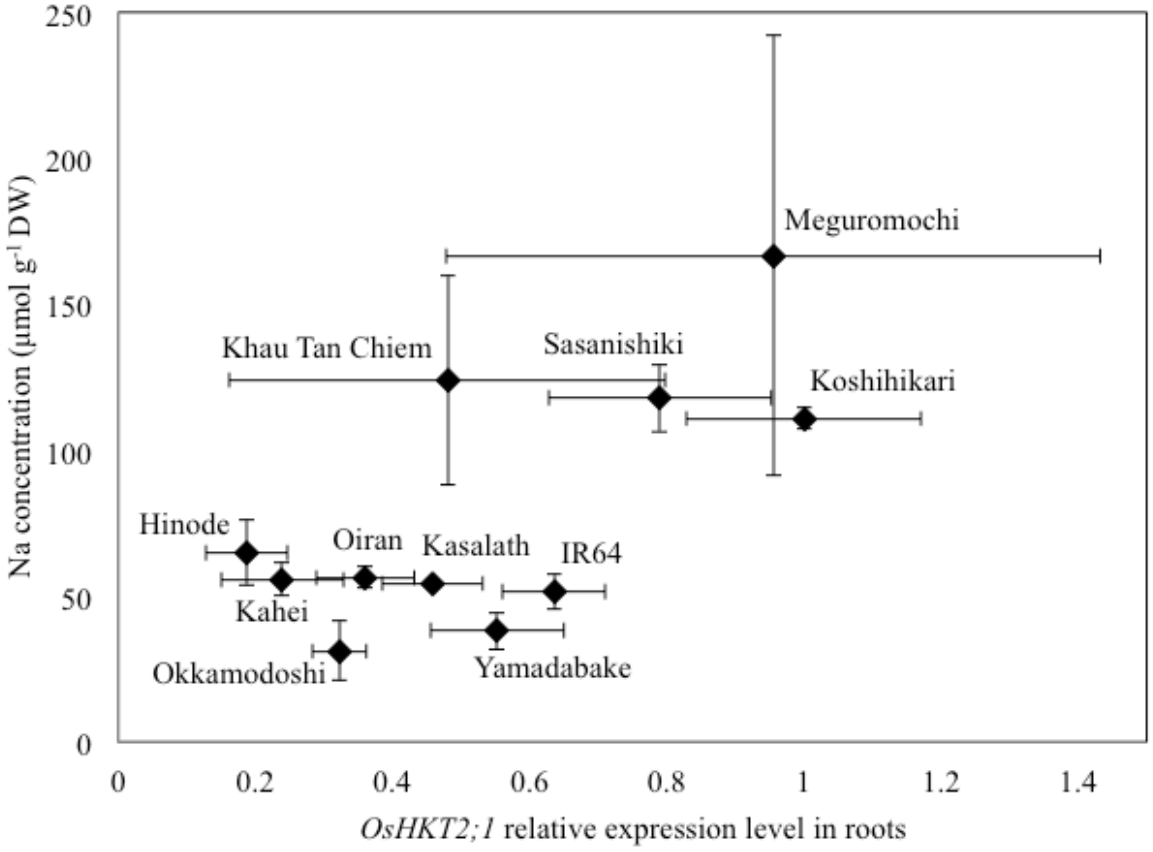
Koshihikari
IR64
87-71

Fig. 2


250
200
150
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50
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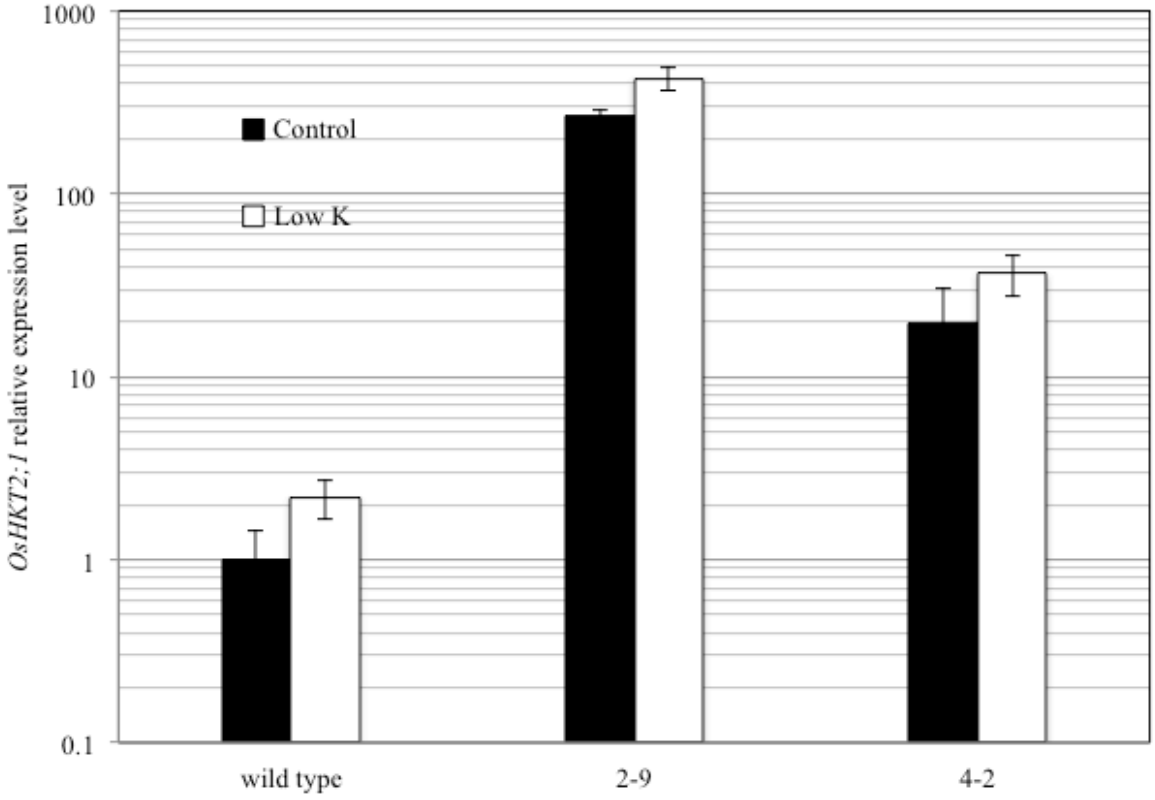
0.8
1
1.2
1.4
OsHKT2;1 relative expression level in roots
Meguromochi
Khau Tan Chiem
Sasanishiki
Koshihikari
Hinode
Oiran
Kasalath
IR64
Kahei
Okkamodoshi
Yamadabake

Fig. 3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0.1
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Control
Low K
wild type
2-9
4-2

Fig. 4

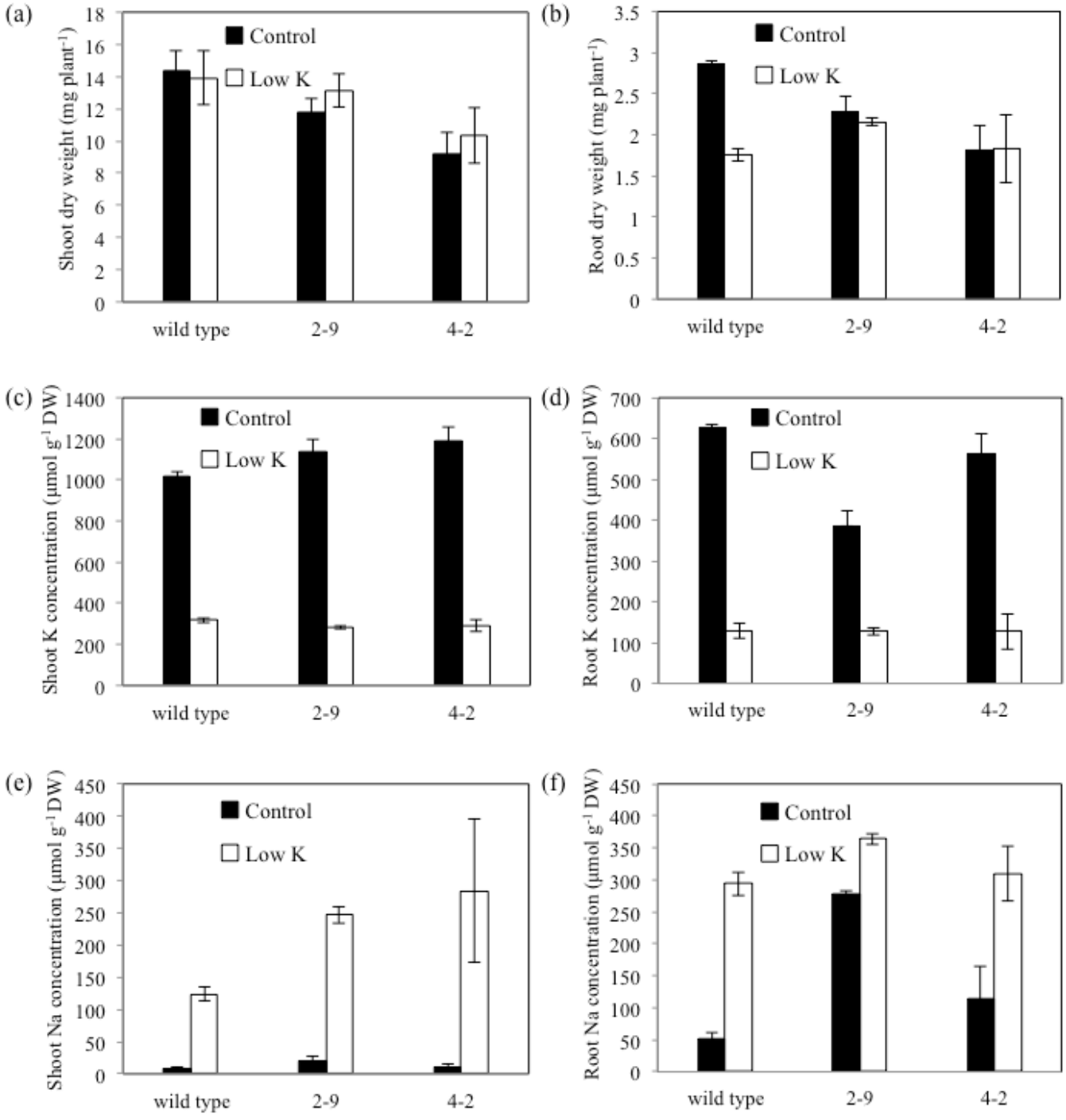
(a)
Shoot dry weight (mg plant⁻¹)
Control
Low K
wild type
2-9
4-2
(b)
Root dry weight (mg plant⁻¹)
Control
Low K
wild type
2-9
4-2
(c)
Shoot K concentration (μmol g⁻¹ DW)
Control
Low K
wild type
2-9
4-2
(d)
Root K concentration (μmol g⁻¹ DW)
Control
Low K
wild type
2-9
4-2
(e)
Shoot Na concentration (μmol g⁻¹ DW)
Control
Low K
wild type
2-9
4-2
(f)
Root Na concentration (μmol g⁻¹ DW)
Control
Low K
wild type
2-9
4-2

Fig. 5