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OsHKT2;2/1-mediated Na$^+$ influx over K$^+$ uptake in roots potentially increases toxic Na$^+$ accumulation in a salt-tolerant landrace of rice Nona Bokra upon salinity stress

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Abstract HKT transporters are Na$^+$-permeable membrane proteins, which mediate Na$^+$ and K$^+$ homeostasis in K$^+$-depleted and saline environments in plants. Class II HKT transporters, a distinct subgroup found predominantly in monocots, are known to mediate Na$^+$-K$^+$ co-transport in principle. Here we report features of ion transport functions of No-OsHKT2;2/1, a class II transporter identified in a salt tolerant landrace of *indica* rice, Nona Bokra. We profiled No-*OsHKT2;2/1* expression in organs of Nona Bokra plants with or without salinity stress. Dominant accumulation of the No-*OsHKT2;2/1* transcript in K$^+$-starved roots of Nona Bokra plants largely disappeared in response to 50 mM NaCl. We found that No-OsHKT2;2/1 expressed in the high-affinity K$^+$ uptake deficient mutant of *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes shows robust K$^+$ selectivity even in the presence of a large amount of NaCl as reported previously. However, No-OsHKT2;2/1-expressing yeast cells exhibited Na$^+$ hypersensitive growth under various concentrations of K$^+$ and Na$^+$ as the cells expressing Po-OsHKT2;2, a similar class II transporter from another salt tolerant *indica* rice Pokkali, when compared with the growth of cells harboring empty vector or cells expressing OsHKT2;4. The OsHKT2;4 protein expressed in *Xenopus* oocytes showed strong K$^+$ selectivity in the presence of 50 mM NaCl in comparison with No-OsHKT2;2/1 and Po-OsHKT2;2. Together with apparent plasma membrane-localization of No-OsHKT2;2/1, these results point to possibilities that No-OsHKT2;2/1 could mediate destructive Na$^+$ influx over K$^+$ uptake in Nona Bokra plants upon salinity stress, and that a predominant physiological function of No-OsHKT2;2/1 might be the acquisition of Na$^+$ and K$^+$ in K$^+$-limited environments.

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Introduction

K$^+$ is one of the three primary macronutrients in plant nutrition and the most abundant monovalent cation in the cytosol of plant cells (Lebaudy et al. 2007). Although K$^+$ is not a component of essential substances such as nucleic acids and proteins as a component like nitrogen and phosphorus, it takes on diverse and vital roles in plant cells, by which the metabolic environment and cellular functions are stably maintained (Gierth and Mäser 2007; Hauser and Horie 2010). In general, plants preferentially accumulate K$^+$ in shoots more than twofold compared to roots and K$^+$

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nutrition has a major influence on shoot growth and the yield of crop plants (Flowers and Läuchli 1983).

K$^+$ nutrition also has a large impact upon salt tolerance of glycophytes. In salt accumulated soil, Na$^+$ is one of the major toxic elements that cause negative effects on the growth and productivity of plants (Deinlein et al. 2014). Na$^+$ over-accumulation outside and inside of plants triggers various toxic effects. High concentration of Na$^+$ in the soil environment decreases water potential, results in prevention of water uptake, disturbs K$^+$ absorption by a competitive inhibition, and causes K$^+$ deficiency (Blumwald 2000; Horie et al. 2012). Over-accumulated Na$^+$ in tissues and cells leads to various toxic effects (Na$^+$ toxicity) on ion homeostasis of essential elements and vital metabolism including photosynthesis with the accumulation of reactive oxygen species (Deinlein et al. 2014; Munns and Tester 2008). Maintenance of higher K$^+$ contents and lower Na$^+$ contents, i.e. high K$^+$/Na$^+$ concentration ratios, in leaves has been demonstrated to be an essential factor for plant salt tolerance (Hauser and Horie 2010; Horie et al. 2009; Munns and Tester 2008). In addition, recent studies have indicated that the ability of K$^+$ retention in the cytosol of leaf mesophyll and root cells is an important trait in the mechanism of salt tolerance of wheat and barley plants (Chen et al. 2005, 2007; Cuin et al. 2008; Wu et al. 2014, 2015).

HKT transporters are functionally related to transport and homeostasis of Na$^+$ and K$^+$, and are identified in many plant species as a gene family (Hauser and Horie 2010; Horie et al. 2009; Munns and Tester 2008). Class I HKT transporters, which are a predominant type of plant HKTs, show more Na$^+$ selective transport. Some of class I HKT (HKT1) transporters have been reported to act as a salt tolerance determinant by promoting xylem Na$^+$ unloading and Na$^+$ exclusion from leaves in Arabidopsis, rice and wheat (Byrt et al. 2007; Davenport et al. 2007; Huang et al. 2006; Hamamoto et al. 2014; Mäser et al. 2002a; Møller et al. 2009; Munns et al. 2012; Ren et al. 2005; Sunarpi et al. 2005; Schroeder et al. 2013). Class II HKT (HKT2) transporters that have been found only in monocots thus far, in general, mediate Na$^+$-K$^+$ co-transport (Gassmann et al. 1996; Haro et al. 2005; Horie et al. 2001; Oomen et al. 2012; Rubio et al. 1995; Schachtman and Schroeder 1994; Yao et al. 2010). Na$^+$ influx in roots mediated by OsHKT2;1, a unique HKT2 transporter in rice that shows distinct Na$^+$/K$^+$ selectivity compared to the typical HKT2 transporters (Garciadeblás et al. 2003; Golldack et al. 2002; Horie et al. 2001; Jabnoune et al. 2009; Oomen et al. 2012; Yao et al. 2010), has been demonstrated to compensate for K$^+$ deficiency and enhance rice growth under K$^+$-restricted conditions (Horie et al. 2007). Po-OsHKT2;2 and No-OsHKT2;2/1, identified in salt tolerant landraces Pokkali and Nona Bokra, are the closest

orthologs of OsHKT2;1 (Horie et al. 2001; Oomen et al. 2012). The No-*OsHKT2;2/1* cDNA isolated from roots of Nona Bokra plants encoded a hybrid protein composed of OsHKT2;1 and Po-OsHKT2;2 (Oomen et al. 2012). No-OsHKT2;2/1 expressed in the K$^+$ uptake-deficient mutant of *S. cerevisiae* and *X. laevis* oocytes maintained a strong permeability to K$^+$ at high external concentrations of Na$^+$ (Oomen et al. 2012). Together with the maintenance of a certain level of expression of No-*OsHKT2;2/1* mRNA in roots imposed to salinity stress, it has been suggested that No-OsHKT2;2/1-mediated robust K$^+$ selectivity in the presence of a large amount of Na$^+$ might contribute to salt tolerant phenotype of Nona Bokra plants (Oomen et al. 2012).

Here, we report a functional study of an independently isolated No-*OsHKT2;2/1*. No-OsHKT2;2/1 showed robust K$^+$ selectivity in heterologous cells even in the presence of a large amount of Na$^+$ as found in the previous study (Oomen et al. 2012). However, No-OsHKT2;2/1 expression caused profound increases in salt sensitivity of a high-affinity K$^+$ uptake deficient mutant of yeast, presumably due to massive Na$^+$ transport activity of the protein over K$^+$ transport. Furthermore, the level of No-OsHKT2;2/1 transcripts was severely decreased in roots of Nona Bokra plants upon salinity stress. We discuss a primary role of the No-OsHKT2;2/1 protein in Nona Bokra plants taking into account the membrane localization of its proteins and features of ion transport and the regulation of its transcript levels during salinity stress.

Materials and methods

Plant material and growth condition

Seeds of Nona Bokra (*Oryza sativa* L. ssp. *indica*) were sterilized as described previously (Horie et al. 2001). For the semi-quantitative RT-PCR analysis, sterilized seeds were germinated and rice seedlings were obtained as described previously (Horie et al. 2001). For the quantitative real time PCR analysis, sterilized seeds were germinated on plastic mesh using a 1 mM CaSO$_4$ solution for 4–5 days and the seedlings were transferred to a plastic pot containing one-half strength of Kimura B nutrient solution (Ma et al. 2001). The solution was changed every 3 days. 50 mM NaCl-included hydroponic solution was treated on 3-week-old plants for 3 days and each organ was harvested. Germination and hydroponic culture were performed using a growth chamber (FLI-301NH, EYELA) at 28 °C on a 14-h light/10-h dark schedule.

Arabidopsis thaliana plants used in this work are of Columbia-0 ecotype. Plants were grown in Jiffy pots (http://www.jiffypot.com/) under long day light period (8 h

light/16 h dark, 150 µmol m^{-2} s^{-1}) at 22 °C and 75 % relative humidity.

Isolation of the No-*OsHKT2;2/1* cDNA

Degenerate primers that are targeted for VPTNENM and SAYGNVG, both of which are relatively well conserved in HKT transporters, were designed (Supplementary Table S1). Genomic PCR yielded an approximately 0.95 kb band that corresponded to a combined sequence of Po-OsHKT2;2 (first half) and OsHKT2;1 (second half). Therefore, primers for Po-OsHKT2;2 and OsHKT2;1 was used to amplify 5′ and 3′ regions of the genomic DNA sequence of No-*OsHKT2;2/1* (Supplementary Table S1). Then, the full length No-*OsHKT2;2/1* cDNA was amplified using primers designed in 5′ and 3′ untranslated regions of the gene (Supplementary Table S1) by the KOD FX polymerase (Toyobo).

Sothern blotting

The genomic DNA was isolated from Nona Bokra seedlings using ISOPLANT (Nippon gene). Approximately 5 µg of genomic DNA was digested with each restriction enzyme, and separated on a 1.0 % (w/v) agarose gel. High stringency hybridization analysis was performed as described previously (Horie et al. 2001).

No-*OsHKT2;2/1* gene expression analysis

Isolation of poly (A)$^{+}$ RNA and the semi-quantitative RT-PCR analysis were performed as described previously (Horie et al. 2001) using root samples obtained from Nona Bokra seedlings grown in 0.3 mM or 30 mM K^{+}-supplemented nutrient solutions. To amplify the No-*OsHKT2;2/1* transcript, a specific primer set was used (Supplementary Table S1).

For the quantitative real time PCR analysis, total RNA was extracted from young leaves (5th and 6th leaf blades and sheathes), basal nodes, and roots. 500 ng of each total RNA was used for reverse transcription and real time PCR was performed using the Thermal Cycler Dice Real Time System II TP800 (Takara). The *OsSMT3* gene was used as an internal control and the same primer set as previous study was used (Horie et al. 2007). Gene specific primers for No-*OsHKT2;2/1* was designed and used (Supplementary Table S1).

Complementation and growth inhibition assays using *S. cerevisiae*

The strain CY162 of *S. cerevisiae* [*MATa*, *Dtrk1*, *trk2::pCK64*, *his3*, *leu2*, *ura3*, *trp1*, *ade2*] was used.

No-*OsHKT2;2/1* cDNA and chimeric *EGFP*-No-*OsHKT2;2/1* DNA fragments were subcloned into the plasmid pYES2 (Invitrogen) and used for yeast transformation with other pYES2::*OsHKT2* cDNA constructs, reported previously (Horie et al. 2001, 2011). Transformants and subsequent growth assays using an AP medium (Rodríguez-Navarro and Ramos 1984) and a general YNB medium were performed as described previously (Horie et al. 2001, 2011). Briefly, for both complementation and Na^{+} sensitivity analyses, indicated concentrations of KCl and NaCl were supplemented in each plate and gene expression was induced by the addition of 2 % (w/v) galactose and 0.6 % (w/v) sucrose. Liquid-cultured cells were washed with the AP medium with no added K^{+} and Na^{+}. 1:10 serial dilutions were prepared for each construct with the maximum OD$_{600}$ of approximately 0.1 and subsequently they were spotted onto each plate. Plates were incubated at 30 °C for 4–6 days.

Two electrode voltage clamp (TEVC) analysis using *X. laevis* oocytes

No-*OsHKT2;2/1* cDNA was subcloned into the pXßG-ev1 vector and the cRNA was transcribed using the mMESSAGE mMACHINE in vitro transcription kit (Ambion®Life Technologies). As for Po-OsHKT2;2 and OsHKT2;4 recordings, previous constructs using pXßG-ev1 were used (Horie et al. 2001, 2011). Oocytes were prepared and TEVC was performed as described previously (Horie et al. 2011; Yao et al. 2010). In brief, 6.25 ng of No-*OsHKT2;2/1* cRNA, 6.25 ng of Po-*OsHKT2;2* cRNA, 50 ng of *OsHKT2;4* cRNA or water was injected into *X. laevis* oocytes for voltage-clamp recordings. Recordings were performed 1 day after injection using an Axoclamp 900A amplifier (Axon Instruments) and an Axon Instruments Digidata 1440A and pCLAMP 10 (Axon Instruments) were used for data analyses. Oocytes were bathed in a solution containing 2 mM NaCl, 6 mM MgSO$_4$, 1.8 mM CaCl$_2$, 10 mM MES-1,3-bis(tris[hydroxymethyl]methylamino) propane, 180 mM D-mannitol, pH 5.5 (with BisTrisPropane) or 50 mM NaCl, 6 mM MgSO$_4$, 1.8 mM CaCl$_2$, 10 mM MES-1,3-bis(tris[hydroxymethyl]methylamino) propane, 90 mM D-mannitol, pH 5.5 (with BisTris-Propane), supplemented with the indicated concentrations of K-gluconate, during the recordings. Voltage steps were applied from +30 to −120 mV in 15-mV decrements with a holding potential of −40 mV. All experiments were performed at room temperature.

Subcellular localization analysis using Arabidopsis mesophyll protoplasts

The Arabidopsis mesophyll protoplasts were isolated and transformed as described in Horie et al. (2011). Briefly,

protoplasts were transformed with 5 µg of DNA for each construct (Free-EGFP and EGFP-No-OsHKT2;2/1) and incubated at 20 °C in the dark for 16 h. For FM4-64 protoplast staining, the dye was directly added to the protoplast suspension with a final concentration of 17 µM for 5 min. Confocal microscope analyses were performed using a Nikon PCM2000 (Bio-Rad, Germany) laser scanning confocal imaging system. The images acquired from the confocal microscope were processed using ImageJ (http://rsbweb.nih.gov/ij/).

Results

Structure of the No-OsHKT2;2/1 protein

A high stringency Southern blotting analysis using Nona Bokra (*Oryza sativa* L. ssp. indica) genomic DNA digested with three different restriction enzymes has revealed a single main band in each lane (Supplementary Fig. S1), suggesting that Nona Bokra plants retain one *OsHKT2* gene highly identical to *OsHKT2;1* unlike the case of a similar salt tolerant landrace Pokkali that retains two similar genes (Horie et al. 2001). A partial sequence of the No-*OsHKT2;2/1* gene was amplified from the genome of Nona Bokra by PCR using a combination of degenerated primers (Supplementary Table S1). Based on this partial sequence, the genomic No-*OsHKT2;2/1* sequence was further determined and the full length cDNA was isolated from the roots of K^+-starved Nona Bokra plants. The No-*OsHKT2;2/1* cDNA (accession number: LC060788) was deduced to encode 530 amino acids composed of the N-terminal half that is highly identical with Po-OsHKT2;2 and the C-terminal half that is identical with OsHKT2;1 (Supplementary Fig. S2a) as found in the previous report (Oomen et al. 2012). Amino acid sequence alignments of No-OsHKT2;2/1 with class II HKT transporters of rice, wheat and barley indicated that the conserved glycine residue that has been proven to be important for robust K^+ selectivity (Mäser et al. 2002b) was conserved in all four filter-pore-forming (p-loop) regions in the putative corresponding domains of No-OsHKT2;2/1 (Supplementary Fig. S2b).

Expression profile of No-*OsHKT2;2/1* in different organs under various ionic conditions

OsHKT2;1 and Po-*OsHKT2;2* genes were reported to be up-regulated in response to K^+ deficiency (Garciadeblás et al. 2003; Horie et al. 2001, 2007; Oomen et al. 2012). Nona Bokra seeds were germinated under conditions of high (30 mM) and low (0.3 mM) K^+-supply and roots were harvested from the seedlings. The level of

No-*OsHKT2;2/1* mRNA in these roots was surveyed by RT-PCR and found to be increased in a low K^+ condition (Fig. 1a). We then prepared 3-week-old plants by hydroponic culture, which were subsequently treated with or without 50 mM NaCl for 3 days. Young leaf blades, leaf sheaths, basal nodes, and roots were separately collected and quantitative real time PCR analyses were performed. Under the control condition (0.18 mM K^+, no added Na^+), the level of No-*OsHKT2;2/1* mRNA in roots was remarkably high when compared with other tissues, which were approximately 48 times higher than that in the 6th leaf blades (Fig. 1b). However, 3d-treatment of 50 mM NaCl led to a substantial reduction in the accumulation of No-*OsHKT2;2/1* mRNA in roots, which was approximately 180 times less than that in control roots (Fig. 1b). Significant reductions in No-*OsHKT2;2/1* transcripts were also found in the 6th leaf sheaths and basal nodes in the presence of 50 mM NaCl (Fig. 1b).
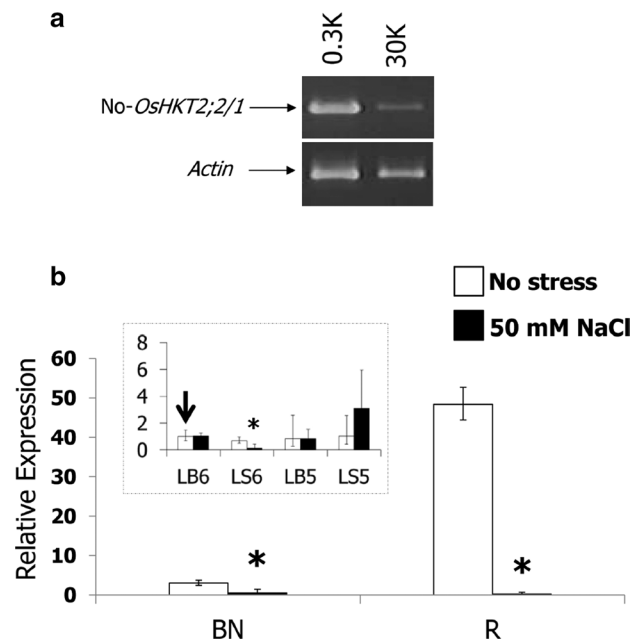

Fig. 1 Expression profiles of No-*OsHKT2;2/1* in Nona Bokra plants grown in various ionic conditions. **a** RT-PCR analysis of the No-*OsHKT2;2/1* transcripts in roots of 4-day-old Nona Bokra seedlings, germinated and grown in N6 medium containing indicated amount of K^+. 100 ng of poly(A)$^+$ RNA prepared from each root sample was used. Actin transcripts were amplified as an internal control. **b** Quantitative real time PCR analysis was performed using tissue samples from approximately 3-week-old Nona Bokra plants grown hydroponically. Total RNA was extracted from leaf blades (LB), leaf sheaths (LS) of the 5th and the 6th young leaves, basal nodes (BN), and roots (R) of Nona Bokra plants treated with or without 50 mM NaCl for 3 days. The level of No-*OsHKT2;2/1* expression was normalized by the level of *OsSMT3* expression (n = 4–6, ±SD). The expression in the 6th leaf blade (LB6) was set to 1 and relative expression of each tissue was shown. *Asterisks* represent a significant difference compared with the control condition (the Welch's-t test: $P < 0.01$)

Fig. 2 Growth assays using high-affinity K$^+$ uptake deficient mutant (strain CY162) of *S. cerevisiae* expressing each OsHKT2 transporter under various ionic conditions. CY162 cells harboring either vector (pYES2) or pYES2::*OsHKT2* plasmids were grown on AP medium containing various concentrations of K$^+$ and Na$^+$ as follows (in mM): **a** 0.1 K$^+$ (no added Na$^+$), **b** 0.1 K$^+$, 30Na$^+$, **c** 0.1 K$^+$, 50Na$^+$, **d** 1 K$^+$ (no added Na$^+$), **e** 1 K$^+$, 30Na$^+$, **f** 1 K$^+$, 50Na$^+$. The cells were also grown on YNB medium containing 7 mM K$^+$ and various concentrations of Na$^+$ as follows (in mM): **g** 7 K$^+$ (no added Na$^+$), **h** 7 K$^+$, 25Na$^+$, **i** 7 K$^+$, 50Na$^+$. 1:10 serial dilutions of each transformant were spotted on plates as described previously (Horie et al. 2011). All plates were incubated at 30 °C for 4–6 days
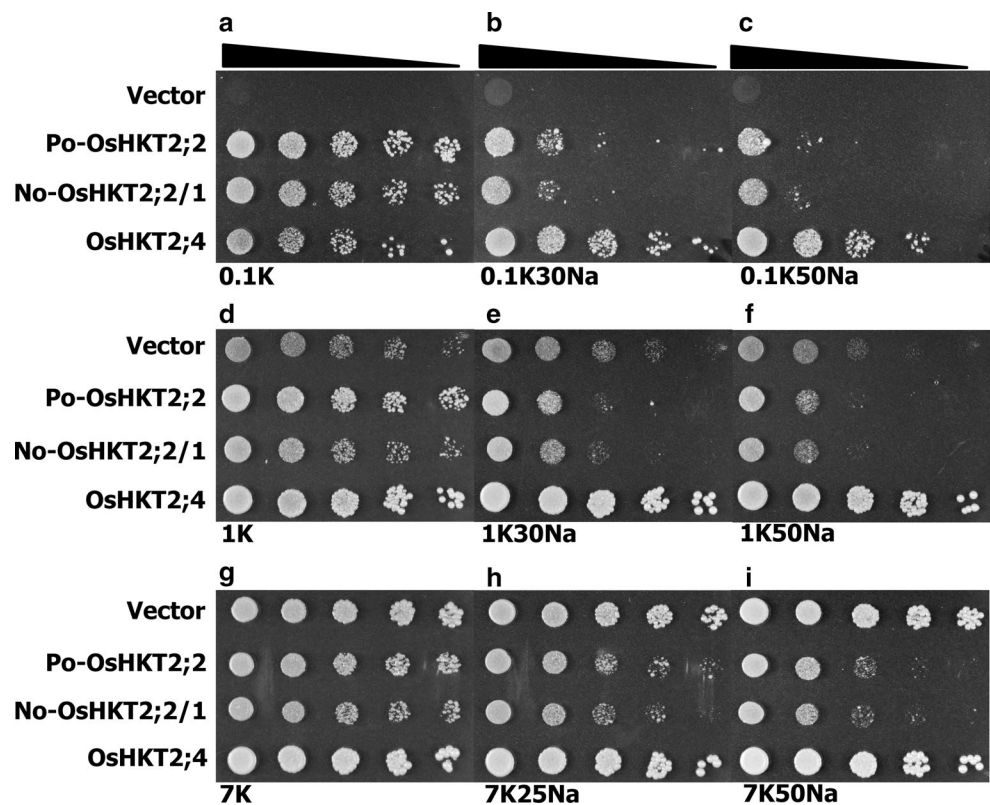
Ion transport properties of No-OsHKT2;2/1 expressed in heterologous expression systems

Dysfunctional mutations in TRK1 and TRK2 high-affinity K$^+$ uptake systems in budding yeast *S. cerevisiae* render the mutant cell hypersensitive to salt stress (Yenush et al. 2002). To evaluate Na$^+$ and K$^+$ transport functions under various K$^+$ and Na$^+$ concentrations, No-OsHKT2;2/1 and K$^+$-transporting OsHKT2 proteins were expressed in the strain CY162 of *S. cerevisiae* disrupted in the *TRK* genes. Expression of No-OsHKT2;2/1 complemented the growth defect of CY162 cells on arginine phosphate (AP) medium containing 0.1 mM K$^+$ in the same way as Po-OsHKT2;2 and OsHKT2;4 (Fig. 2a). Increases in the NaCl concentration, however, triggered salt sensitive growth phenotype of CY162 cells expressing Po-OsHKT2;2 and No-OsHKT2;2/1 in the presence of 0.1 mM K$^+$ while cells expressing OsHKT2;4, which exhibits relatively low Na$^+$ transport activity did not show sensitivity to Na$^+$ addition (Fig. 2a–c; Horie et al. 2011; Lan et al. 2010; Sassi et al. 2012). An increase in K$^+$ concentration from 0.1 mM to 1 mM did not bring about noticeable improvement in the growth of No-OsHKT2;2/1- and also Po-OsHKT2;2-expressing cells during salt stress, which showed more Na$^+$ sensitive growth when compared with vector-control cells (Fig. 2d–f). We further used yeast nitrogen base (YNB) medium richer than AP medium for an additional growth

assay. In the presence of 7 mM K$^+$, Na$^+$ sensitivity of the No-OsHKT2;2/1 and the Po-OsHKT2;2-expressing cells reduced to some extent in comparison with the cells grown in AP medium (Fig. 2b, c, e, f, h, i). However, these cells still showed more severe Na$^+$ sensitivity than vector-control cells (Fig. 2g–i).

We next analyzed features of OsHKT2-mediated Na$^+$ and K$^+$ transport by two electrode voltage clamp (TEVC) experiments using *Xenopus laevis* oocytes. In the presence of 2 mM and 50 mM NaCl, No-OsHKT2;2/1-expressing oocytes elicited large ionic currents clearly distinguishable from currents derived from water-injected oocytes (Fig. 3a, b). Increasing the Na$^+$ concentration from 2 to 50 mM led to significant positive shifts in the reversal potential of No-OsHKT2;2/1-expressing oocytes (Fig. 3b). Addition of 0.1 mM K-gluconate salt in the 50 mM Na$^+$-containing bath solution influenced neither on the overall currents of No-OsHKT2;2/1-expressing oocytes (Fig. 3a) nor on reversal potentials (Fig. 3b). In contrast, increases in the K$^+$ concentration up to 10 mM noticeably amplified No-OsHKT2;2/1-mediated currents (Fig. 3a). The current amplitude mediated by No-OsHKT2;2/1 at -120 mV in the 50 mM Na$^+$ with 1 mM or 10 mM K$^+$ bath were significantly larger than those in the 50 mM Na$^+$ bath (-12.5 ± 0.9 or -20.8 ± 1.1 µA vs. -7.8 ± 0.5 µA, $P < 0.01$; Fig. 3b). In addition, the reversal potential of No-OsHKT2;2/1-expressing oocytes shifted toward more
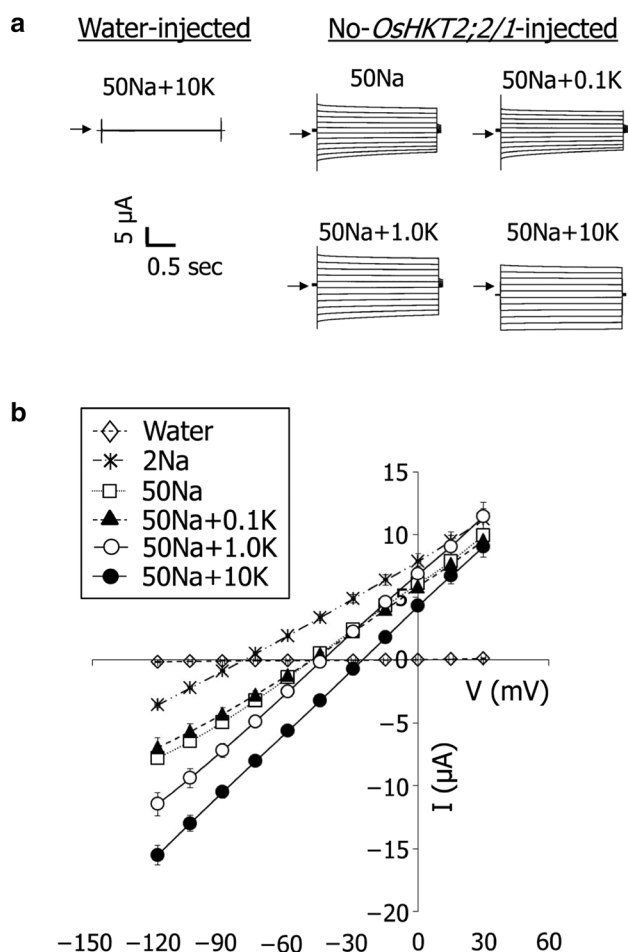
Fig. 3 Two electrode voltage clamp analysis using *X. laevis* oocytes expressing No-OsHKT2;2/1. **a** Representative currents of water- and No-*OsHKT2;2/1* cRNA-injected oocytes bathed in solutions containing indicated amount of NaCl and K-gluconate. Zero current levels are shown by *arrows*. **b** Average current–voltage curves from No-OsHKT2;2/1-expressing oocytes bathed in solutions containing indicated amount of NaCl and K-gluconate (n = 4–6, ±SE). Each *number* represents mM concentrations of Na^+ and K^+ in the solution. Voltage steps ranged from −120 to +30 mV with 15-mV decrements. Note that water-injected oocytes showed similar performance in each solution and thus the result from the condition of 50 mM Na^+ and 10 mM K^+ was presented as representative data

depolarized status in accordance with increases in the K^+ concentration (Fig. 3b), suggesting robust K^+ selectivity of No-OsHKT2;2/1 in the presence of 50 mM Na^+.

Po-OsHKT2;2 exhibited a similar profile of current–voltage relationships to that of No-OsHKT2;2/1 upon increasing K^+ concentrations in the presence of 50 mM Na^+ in oocytes. In comparison with water-injected oocytes that showed small background currents, Po-OsHKT2;2 elicited large outward and inward currents with positive shifts in the reversal potential following increases in the external K^+ concentration (Fig. 4a, b). Current voltage relationships from OsHKT2;4-expressing oocytes, obtained in
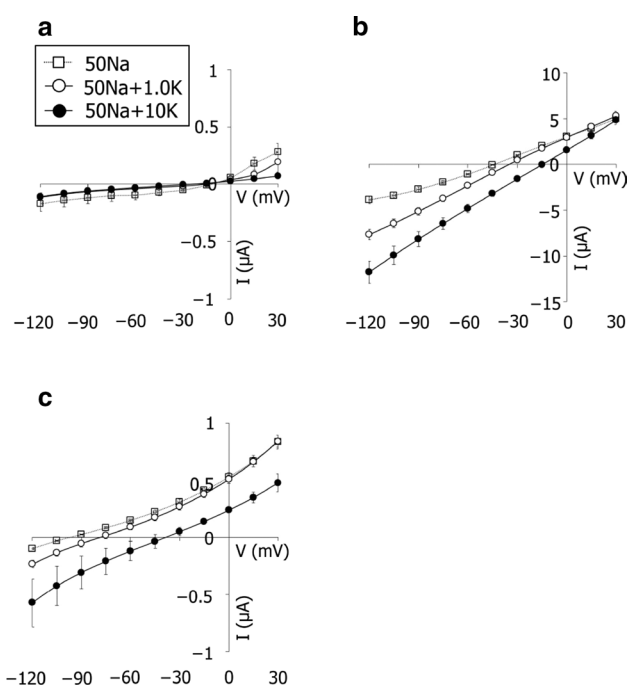

Fig. 4 Two electrode voltage clamp analysis using *X. laevis* oocytes expressing Po-OsHKT2;2 and OsHKT2;4. **a** Average current–voltage curves from water-injected control oocytes bathed in solutions containing indicated amount of NaCl and K-gluconate (n = 6, ±SE). Each *number* represents mM concentrations of Na^+ and K^+ in the solution. **b** Average current–voltage curves from Po-OsHKT2;2-expressing oocytes bathed in the same solutions as control oocytes (n = 6, ±SE). **c** Average current–voltage curves from OsHKT2;4-expressing oocytes bathed in the same solutions as control oocytes (n = 6, ±SE). Voltage steps ranged from −120 to +30 mV with 15-mV decrements

the same condition, also indicated K^+-dependent positive shifts in the reversal potential in the presence of 50 mM Na^+ although overall currents elicited by OsHKT2;4 were much smaller than those from No-OsHKT2;2/1 and Po-OsHKT2;2 (Figs. 3b, 4b, c), which is consistent with the previous findings (Horie et al. 2011).

Subcellular localization of the EGFP-fused No-OsHKT2;2/1 protein in plant cells

The *OsHKT2;2/1* cDNA fused in frame with the enhanced green fluorescence protein (EGFP) cDNA at the N-terminal side was constructed to investigate subcellular localization of OsHKT2;2/1 in plant cells. EGFP-No-OsHKT2;2/1 or EGFP was transiently expressed in protoplasts prepared from *Arabidopsis thaliana* mesophyll cells. Confocal microscopy analyses have revealed that strong EGFP fluorescence can be observed in the periphery of mesophyll protoplasts expressing EGFP-No-OsHKT2;2/1 (Fig. 5a). The green fluorescence clearly overlapped with the red fluorescence from the FM4-64 dye, a plasma membrane

Fig. 5 Subcellular localization of the EGFP-fused No-OsHKT2;2/1 chimeric protein in Arabidopsis mesophyll protoplasts. 35S promoter-driven *EGFP*-No-*OsHKT2;2/1* or *EGFP* was transiently expressed in the mesophyll protoplasts. **a** Green fluorescence from EGFP-No-OsHKT2;2/1. **b** Red fluorescence from FM4-64 labeled membrane and chlorophyll autofluorescence (CHL) in EGFP- No-OsHKT2;2/1-expressing cells. **c** Overlay image of **a** and **b**. **d** Green fluorescence from the free EGFP. **e** FM4-64 and chlorophyll autofluorescence (CHL)-derived red fluorescence in EGFP-expressing cells. **f** Overlay image of **d** and **e**

EGFP   FM4-64/CHL   Overlay

Bar = 10 μm
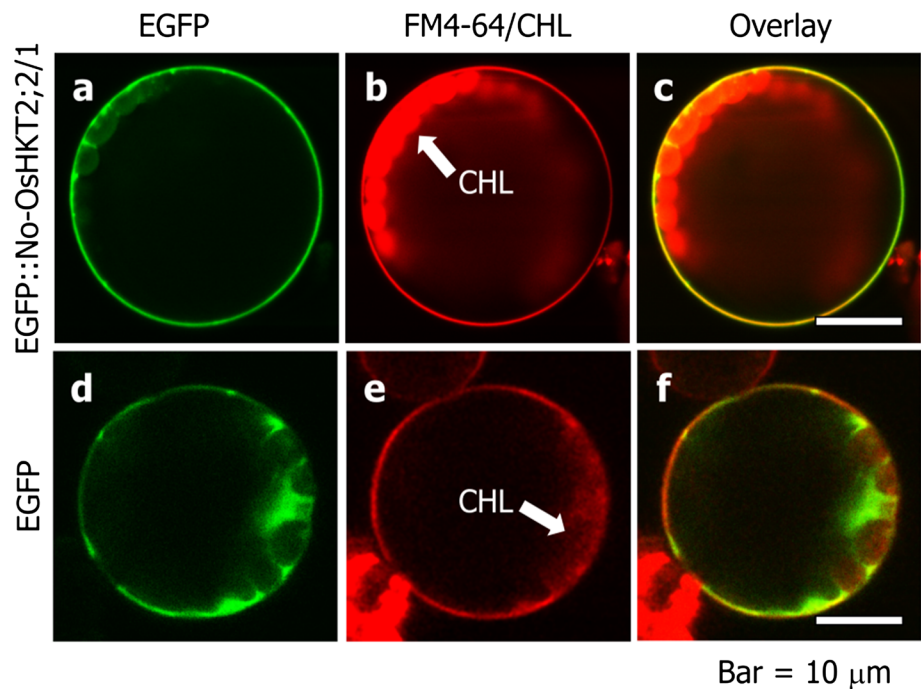
marker (Bolte et al. 2004) (Fig. 5b, c), indicating that EGFP-No-OsHKT2;2/1 localizes to the plasma membrane. Note that the EGFP-fused No-OsHKT2;2/1 protein was found to be functional when expressed in the high-affinity K$^+$ uptake deficient mutant of yeast (Supplementary Fig. S3). Transient expression of the control EGFP in mesophyll cells produced green fluorescence inside the cell, which does not overlap with FM4-64-derived red fluorescence at the cell periphery (Fig. 5d–f).

Discussion

HKT transporters are closely relevant to Na$^+$ and K$^+$ transport and homeostasis in plants (Horie et al. 2009). Based on the sequences and ion transport functions, HKT proteins can be divided into at least two or potentially three subgroups (class I and II or I–III: Hauser and Horie 2010).

Nine *OsHKT* genes have been identified in a japonica rice cultivar Nipponbare with the two of them being pseudogenes (Garciadeblás et al. 2003). In fact, the *OsHKT2;2* gene, which correspond to Po-*OsHKT2;2* isolated from a salt tolerant indica landrace Pokkali, is one of the two pseudogenes in Nipponbare and has a large deletion on the genome (Garciadeblás et al. 2003; Horie et al. 2001). OsHKT2;1 and Po-OsHKT2;2 were the closest ortholog among class II HKTs (Hauser and Horie 2010). The No-*OsHKT2;2/1* cDNA isolated from another famous salt tolerant landrace Nona Bokra was found to encode a hybrid protein consists of OsHKT2;1 and Po-OsHKT2;2 (Supplementary Fig. S2a), consistent with the previous study

(Oomen et al. 2012). No-OsHKT2;2/1-mediated ion transport and a possible physiological role of the protein in the mechanism of salt tolerance of Nona Bokra plants have been reported prior to this study (Oomen et al. 2012). However, we had also isolated the same cDNA and carried out our own research regarding the structure and function of the No-OsHKT2;2/1 transporter from Nona Bokra plants.

Structure and K$^+$ selectivity of the No-OsHKT2;2/1 protein

The p-loop hypothesis regarding the selectivity-pore-forming region has been proposed, in which HKT/Trk/Ktr proteins from fungi to plants hold four filter-pore-forming domains (p-loops) sandwiched by transmembrane domains, which is called an MPM motif (Supplementary Fig. 2b; Durell and Guy 1999; Durell et al. 1999; Hauser and Horie 2010; Kato et al. 2001; Mäser et al. 2002b; Tholema et al. 2005). In each p-loop domain, a conserved glycine (Gly) residue, which is distantly homologous to the first Gly in the highly conserved GYG sequence determining K$^+$ selectivity of the shaker type K$^+$ channel, was reported to play a key role in K$^+$ selectivity of the HKT/Trk/Ktr family (Supplementary Fig. S2b; Mäser et al. 2002b; Tholema et al. 2005). OsHKT2;1 is a unique class II HKT transporter retaining a serine (Ser) residue at the conserved Gly position in the first p-loop (Supplementary Fig. S2; Hauser and Horie 2010) and it has been shown that OsHKT2;1 lacks high-affinity K$^+$ uptake activity when expressed in yeast and tobacco BY2 cells (Horie et al. 2001; Yao et al. 2010). Since No-OsHKT2;2/1 consists of the N-terminal half

of Po-OsHKT2;2 and the C-terminal half of OsHKT2;1, all four Gly residues in p-loops are retained as other typical class II transporters that mediate Na^+-K^+ co-transport (Supplementary Fig. S2), consistent with the previous report (Oomen et al. 2012). Therefore, the domain structure of p-loops in No-OsHKT2;2/1 suggests it mediates high-affinity K^+ uptake similar to TaHKT2;1 and Po-OsHKT2;2. Consistently, No-OsHKT2;2/1 rescued the growth of the CY162 cell that is deficient in high-affinity K^+ uptake in the presence of 0.1 mM K^+ (Fig. 2a). Po-OsHKT2;2 constitutively expressed in tobacco BY2 cells has been proven to mediate high-affinity K^+ transport by the direct tracer influx experiments and ICP analyses (Yao et al. 2010). Together with the highly accumulated transcripts of the No-*OsHKT2;2/1* gene in roots of seedlings and 3-week-old plants in low K^+ conditions (Fig. 1), our results support the possibility that No-OsHKT2;2/1 functions as a high-affinity K^+ transporter in Nona Bokra plants. However, it should be noted that high-affinity K^+ transport activity mediated by typical HKT2 transporters has not yet been robustly proven *in planta*. Transgenic wheat plants harboring the antisense *TaHKT2;1* construct were reported to exhibit no difference in low K^+-stimulated depolarization of root cortical cells in the transition from the K^+-depleted buffer when compared with the control plants, indicating that a reduction in the *TaHKT2;1* expression had no effect on the overall K^+ absorption in roots under the low K^+ condition (Laurie et al. 2002). Moreover, a similar Na^+-K^+ co-transporter of barley HvHKT2;1 also did not provide strong evidence for K^+ transport when overexpressed in barley plants against the fact that HvHKT2;1 shows robust activity for K^+ transport in heterologous expression systems (Mian et al. 2011). A possible explanation would be that the effect of an increase or decrease in the HKT2-mediated K^+ transport activity could be masked by multiple cell layers of the root and shoot with multiple K^+ transport systems. Notably, argument over the presence of Na^+-driven K^+ uptake activity in higher terrestrial plants has been raised (Maathuis et al. 1996; Walker et al. 1996). Thus, whether typical class II HKT transporters mediate Na^+-K^+ co-transport activity *in planta* remains an important open question.

Role of No-OsHKT2;2/1 in Nona Bokra plants

K^+ and Na^+ are alkali cations that show similar chemical properties. However, impacts of each cation on plant nutrition and plant salt tolerance are fairly different. K^+ is an essential macronutrient with diverse vital roles in plant cells (Hauser and Horie 2010). Glycophytes, including most of agricultural plants, prefer high K/Na concentration ratios in shoots especially in leaves under salinity stress to maintain a stable metabolic environment and various

cellular functions (Hauser and Horie 2010). In wheat and barley plants, the ability to retain K^+ in the cytosol of leaf mesophyll cells has been indicated to be an essential trait against salinity stress (Wu et al. 2014, 2015). Salt-induced K^+ loss was found to promote the production of reactive oxygen species (ROS) and ROS-induced programmed cell death in root epidermal and leaf mesophyll cells of wheat and barley plants (Chen et al. 2007; Cuin et al. 2008; Wu et al. 2014, 2015). Therefore, maintenance of acquisition and distribution of K^+ during salinity stress has capacity to enhance overall salt tolerance of the plants.

Based on the robust K^+ selectivity of No-OsHKT2;2/1 expressed in heterologous cells, an intriguing hypothesis for a possible physiological role of No-OsHKT2;2/1 has been proposed, in which No-OsHKT2;2/1 was suggested to contribute to salt tolerant phenotype of Nona Bokra plants due to the No-OsHKT2;2/1-mediated K^+ acquisition during salinity stress (Oomen et al. 2012). Our heterologous expression analysis also indicated that No-OsHKT2;2/1 maintains K^+ selectivity in the presence of a large amount of NaCl (Figs. 2a–c, 3). Addition of 1 mM and 10 mM K^+ in the 50 mM Na^+ solution led to shifts in the reversal potential of No-OsHKT2;2/1-expressing oocytes toward depolarized status with increases in the amplitude of No-OsHKT2;2/1-mediated currents, indicating that No-OsHKT2;2/1 mediates K^+ transport in a mM concentration range as well, even when a high concentration of Na^+ coexists (Fig. 3). This result is consistent with the one reported previously (Oomen et al. 2012), together the results suggest that No-OsHKT2;2/1 could maintain K^+ acquisition in salt-stressed Nona Bokra roots if it is expressed. In fact, expression of No-OsHKT2;2/1 and Po-OsHKT2;2 in the high-affinity K^+ uptake mutant of yeast was reported to maintain K^+ uptake in the presence of 0.05 mM K^+ and 100 mM Na^+ (Oomen et al. 2012). As shown in Fig. 2, however, our growth assays using transformants of CY162 cells revealed that No-OsHKT2;2/1 expression as a whole rendered the mutant cells Na^+ hypersensitive, similar to the case of Po-OsHKT2;2 expression. OsHKT2;4 has been found to mediate K^+ transport with relatively low Na^+ transport activity when compared with the other HKT2 transporters such as Po-OsHKT2;2 (Horie et al. 2011; Sassi et al. 2012). OsHKT2;4-expressed CY162 cells exhibited firm growth and NaCl-resistant phenotype in salt stress conditions tested in comparison with other transformants presumably due to stable acquisition of K^+ with less Na^+ uptake via OsHKT2;4 (Fig. 2). In addition to the expression analysis using yeast, electrophysiological measurements revealed that No-OsHKT2;2/1 and Po-OsHKT2;2 show a similar transport profile including the sensitivity to the extracellular K^+ (Figs. 3b, 4b). Interestingly, increased extracellular K^+ concentrations stimulated not only inward currents but also

outward currents in No-OsHKT2;2/1- and Po-OsHKT2;2-expressing oocytes, which, in contrast, was not the case in OsHKT2;4-expressed oocytes (Figs. 3b, 4b, c). These results might be an indication of K^+-induced transport activation of No-OsHKT2;2/1 and Po-OsHKT2;2. Given that the expression of No-OsHKT2;2/1 and Po-OsHKT2;2 in yeast cells increased salt sensitivity even in the presence of 7 mM K^+ condition (Fig. 2g–i), it is important to examine whether the extracellular K^+ enhances Na^+ influx mediated by No-OsHKT2;2/1 and Po-OsHKT2;2 in future research. Furthermore, upon the addition of 10 mM K^+, OsHKT2;4-expressing oocytes exhibited larger shifts of the zero current potential toward more depolarized status in comparison with those of No-OsHKT2;2/1 and Po-OsHKT2;2, suggesting strong K^+ selectivity of OsHKT2;4 in the presence of a large amount of Na^+ (Figs. 3b, 4b, c), which supports more salt tolerant phenotype of OsHKT2;4-expressing yeast cells than No-OsHKT2;2/1- and Po-OsHKT2;2-expressing cells under salinity stress (Fig. 2). Taken together, our results imply that No-OsHKT2;2/1 could also mediate destructive Na^+ influx in roots under salinity stress independently of external K^+ concentrations, overwhelming the positive effect of K^+ uptake, and that No-OsHKT2;2/1-expression is unlikely to contribute to the maintenance of high K/Na ratios in the cytosol and thus salt tolerance at the single cell level. Inconsistent with the previous findings, in which a significant level of No-OsHKT2;2/1 expression was maintained even in salt-stressed roots of Nona Bokra plants (Oomen et al. 2012), the imposition of 50 mM NaCl stress for 3 days upon Nona Bokra plants in the vegetative growth period triggered approximately 180 times reductions of the level of No-*OsHKT2;2/1* transcripts in roots when compared with that of non-stress condition (Fig. 2b), supporting the hypothesis above.

OsHKT2;1 is a closest ortholog of No-OsHKT2;2/1 and Po-OsHKT2;2 among plant HKT2 transporters and shows more Na^+ selective transport attributed to the unique p-loop structure (Supplementary Fig. 2b; Horie et al. 2001; Mäser et al. 2002b; Yao et al. 2010). OsHKT2;1 has been proven to mediate Na^+ influx in roots and Na^+ distribution to shoots of rice plants to compensate K^+ deficiency localizing in the plasma membrane (Horie et al. 2007). Furthermore, both the ion transport activity of OsHKT2;1 and the level of *OsHKT2;1* transcript were rapidly down-regulated in response to 30 mM NaCl (Horie et al. 2007). These findings have led to the model that OsHKT2;1 contributes to nutritional Na^+ absorption and distribution in rice plants grown in K^+-depleted environments (Horie et al. 2007). EGFP-fused No-OsHKT2;2/1 clearly localized in the plasma membrane of protoplasts derived from Arabidopsis leaves and rice leaf sheaths (Fig. 5; data not shown). In addition, K^+ deprivation-induced accumulation of No-*OsHKT2;2/1* transcripts was largely vanished in response to salinity stress (Fig. 1). The level of Po-*OsHKT2;2* transcripts have also been found to reduce significantly under salinity stress (Horie et al. 2001; Oomen et al. 2012). These characteristics are fairly similar to those of OsHKT2;1 mentioned above.

Based on our current findings, we suppose that the primary role of No-OsHKT2;2/1 is compensation for K^+ shortage through its Na^+-K^+ co-transport function, and that HKT2 transporters such as Po-OsHKT2;2, TaHKT2;1 and HvHKT2;1, which belong to the same clade with OsHKT2;1 in phylogenetic analyses (Hauser and Horie 2010; Horie et al. 2009), share a similar physiological function. It is an important observation that the down regulation of the expression of the *TaHKT2;1* gene, which encodes a robust Na^+-K^+ co-transporter similar to No-OsHKT2;2/1, caused relatively salt tolerant growth phenotypes of the transgenic wheat plants when compared with the control plants. HvHKT2;1 overexpression has led to enhancement of growth of transgenic barley plants under salinity stress not because of increased K^+ acquisition but because of combinational effects of increased Na^+ influx and distribution and the distinct character of barley as a Na^+ accumulator (Mian et al. 2011). It should be noted, however, that our results do not exclude a potential contribution of No-OsHKT2;2/1 to salt tolerance of Nona Bokra plants. To obtain more direct evidence for the issue, genetic studies using proper plant materials are indispensable. The chromosome segment substitution lines (CSSLs) using Nona Bokra and a japonica rice cultivar Koshihikari as the donor and recipient plants, respectively, has been developed (Takai et al. 2007). Investigating such CSSLs with or without the No-*OsHKT2;2/1* locus under salinity stress will be a powerful strategy to directly evaluate the contribution of the No-*OsHKT2;2/1* gene to salt tolerance. Alternatively, generation and investigation of transgenic rice plants such as No-*OsHKT2;2/1*-harboring japonica cultivars and No-*OsHKT2;2/1* RNAi Nona Bokra plants will also be helpful.

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