HKT2;2/1, a $K^+$-permeable transporter identified in a salt-tolerant rice cultivar through surveys of natural genetic polymorphism

Ronald J.F.J. Oomen, Begoña Benito, Hervé Sentenac, Alonso Rodríguez-Navarro, Manuel Talón, Anne-Aliénor Véry and Concha Domingo

SUMMARY

We have investigated OsHKT2;1 natural variation in a collection of 49 cultivars with different levels of salt tolerance and geographical origins. The effect of identified polymorphism on OsHKT2;1 activity was analysed through heterologous expression of variants in Xenopus oocytes. OsHKT2;1 appeared to be a highly conserved protein with only five possible amino acid substitutions that have no substantial effect on functional properties. Our study, however, also identified a new HKT isoform, No-OsHKT2;2/1 in Nona Bokra, a highly salt-tolerant cultivar. No-OsHKT2;2/1 probably originated from a deletion in chromosome 6, producing a chimeric gene. Its 5′ region corresponds to that of OsHKT2;2, whose full-length sequence is not present in Nipponbare but has been identified in Pokkali, a salt-tolerant rice cultivar. Its 3′ region corresponds to that of OsHKT2;1. No-OsHKT2;2/1 is essentially expressed in roots and displays a significant level of expression at high Na$^+$ concentrations, in contrast to OsHKT2;1. Expressed in Xenopus oocytes or in Saccharomyces cerevisiae, No-OsHKT2;2/1 exhibited a strong permeability to Na$^+$ and K$^+$, even at high external Na$^+$ concentrations, like OsHKT2;2, and in contrast to OsHKT2;1. Our results suggest that No-OsHKT2;2/1 can contribute to Nona Bokra salt tolerance by enabling root K$^+$ uptake under saline conditions.

Keywords: HKT transporter, SNP, rice, salt tolerance.

INTRODUCTION

Analyses of natural genetic polymorphism can provide insight into the mechanisms of plant adaptation to environmental conditions (e.g. soil conditions; Macnair, 1993; Brady et al., 2005; Baxter et al., 2010). One example of such genetic adaptation to soil conditions is provided by the differences in salt tolerances between the Oryza sativa (rice) cultivars Indica and Japonica. Even though O. sativa is considered a salt-sensitive species, several varieties display a certain level of salt tolerance. The majority of these tolerant varieties, which include breeding varieties but also traditional land races, belongs to the Indica subfamily, and are mostly identified in coastal areas (Akbar et al., 1972; Flowers and Yeo, 1981; Asch et al., 2000). Amongst the best-known examples of such varieties are Nona Bokra from India and Pokkali from Sri Lanka. In the Japonica subfamily, Nipponbare, the most studied variety is salt sensitive.

This diversity between rice varieties has allowed the identification of a large number of rice quantitative trait loci (QTLs) linked with salt tolerance. Several QTLs, identified in crosses between salt-tolerant and salt-sensitive rice (Ren et al., 2005) or Triticum spp. (wheat; Huang et al., 2006; Byrt et al., 2007) cultivars, have been shown to correspond to HKT genes, which encode ion transporters permeable to K$^+$ and/or Na$^+$. The OsHKT1;5 gene corresponds to a QTL identified in a Nona Bokra × Koshihikari mapping population, and was shown to play an important role in Na$^+$ and K$^+$ homeostasis in rice leaves (Ren et al., 2005). It has also been shown that HKT genes are significantly involved in salt...
tolerance in other plants. For instance, in Arabidopsis, AthKT1;1, which is permeable to Na⁺ only (Ouzumi et al., 2000), contributes to salt tolerance by unloading Na⁺ (‘desalination’) from the ascending xylem sap, and probably loading this cation into the descending phloem sap, thus limiting Na⁺ levels in the shoots (Berthomieu et al., 2003; Sunarpi et al., 2005; Davenport et al., 2007). Furthermore, polymorphism in the promoter region of AthKT1;1 has been shown to affect AthKT1;1 expression levels and salt tolerance of the accession (Baxter et al., 2010). In Hordeum vulgare (barley), overexpression of HvHKT2;1, which is permeable to both K⁺ and Na⁺, has recently been shown to result in increased salt tolerance (Mian et al., 2011).

Whereas only one HKT is present in Arabidopsis thaliana, between seven and nine paralogues have been identified in rice (Garcidiegibás et al., 2003). The different rice HKTs can be sorted into two subfamilies based on phylogenetic analysis, and in agreement with their ion transport capacity. Subfamily-1 members are only permeable to Na⁺ and subfamily-2 members are permeable to Na⁺ and K⁺ (Corratgé-Faille et al., 2010). In this study, we have focused on OsHKT2;1 from rice and searched for single nucleotide polymorphisms (SNPs) by a tilling approach. By using a selection of rice cultivars with varying levels of salt tolerance, we aimed at identifying SNPs in the OsHKT2;1 gene that might explain the difference in the cultivar's salt tolerance. OsHKT2;1 is weakly permeable to K⁺ and has been shown to be involved in root Na⁺ uptake in Nipponbare (Horie et al., 2001, 2007; Golldack et al., 2002; Garciadiegibás et al., 2003). Interestingly, OsHKT2;2, which is a close homologue of OsHKT2;1 present in the salt-tolerant Pokkali cultivar, but absent in Nipponbare, is more permeable to K⁺ (Horie et al., 2001), a property that may confer to this system a role in Pokkali salt tolerance.

We present an SNP analysis of natural variations of OsHKT2;1 in 49 rice cultivars from different origins. Several variants were identified and tested for their Na⁺ and K⁺ transport characteristics by expression in Xenopus oocytes. The study also led to the identification of a new rice HKT gene in the highly salt tolerant cultivar Nona Bokra. We have studied the transport properties of this new Nona Bokra HKT by heterologous expression in Xenopus oocytes and Saccharomyces cerevisiae. We discuss a putative role of Nona Bokra HKT in salt tolerance.

RESULTS

Sequence polymorphism in OsHKT2;1 in cultivated rice

The OsHKT2;1 coding region sequence was examined by EcoTilling (Raghavan et al., 2007) from a core collection of 49 genetically diverse cultivars. Primers were designed to amplify two amplicons, together covering the complete genomic sequence of OsHKT2;1. Amplified DNA fragments identified to contain polymorphism were further sequenced allowing the detection of nine SNPs when compared with the sequence of the Nipponbare allele (Figure 1a). Five SNPs were non-synonymous substitutions. Six OsHKT2;1 protein variants were finally identified, according to different combinations of the SNPs (Figure 1a; Table S1). The Nipponbare allele was the most predominant among the cultivars analyzed. Variants were found in one or two cultivars regardless of the subfamily, indica or japonica. G17/V and G17/V/D403/ E were present in GIZA178 and Moroberekan cultivars, respectively, both originating from Africa, whereas the rest of the variants R21/K R32/K cv Kasalath and LTH, R21/ K R32/K D403/E (cv IR58), F61/S (cv Habataki) and D403/E (cv Co39) were present in cultivars from Asia. Based on crystal structure analysis of the KcsA bacterial K⁺ channel and multiple sequence alignments showing conservation of residues, a topology displaying four successively arranged transmembrane–pore loop–transmembrane (MTP) domains has been proposed for the transporters of the bacterial Ktr/
fungal Trk/plant HKT superfamily (Doyle et al., 1998; Durell et al., 1999). The proposed topology was further supported by biochemical analyses (Kato et al., 2001) and by the recent crystallization of a bacterial member of the KtR/Trk/HKT superfamily (Cao et al., 2011). According to Durell et al.’s model, the mutations G17/V, R21/K and R32/K are positioned in the N-terminal cytosolic region, and F61/S and D403/E are located at the end of the first and sixth transmembrane segment, respectively (Figure 1b).

**Transport activity of the OsHKT2;1 variants**

Site-directed mutagenesis was performed on Nipponbare OsHKT2;1 cDNA (Ni-OsHKT2;1) to introduce variations at each of the identified positions in the six variants. Variant transporters were expressed in *Xenopus* oocytes and functionally compared with Ni-OsHKT2;1. Ni-OsHKT2;1, when expressed in *Xenopus* oocytes, behaves as a Na⁺-K⁺ symporter at very low concentrations of Na⁺ and K⁺, and as a Na⁺ uniport at Na⁺ concentrations in the millimolar range (Jabnoun et al., 2009). Ni-OsHKT2;1 permeability to both Na⁺ and K⁺ at low concentrations is attested by positive shifts of current-voltage (I–V) relationships along the voltage axis when the concentration of one of the two ions is fixed at submillimolar concentration and the concentration of the other ion increases (Figure 2a,c,f,h). At high Na⁺ concentrations OsHKT2;1 does not transport K⁺ any more. The dependency of Na⁺:K⁺ transport stoichiometry on external Na⁺ is revealed by the increase in the slope of the OsHKT2;1 reversal potential versus Na⁺ activity (Figure 2f). Another typical feature of OsHKT2;1 is a strong sensitivity (block) to external K⁺ (Figure 2g).

The functional properties of Ni-OsHKT2;1 were compared with those of the six OsHKT2;1 ectopic variants identified (Figure 2). No significant difference was observed in terms of macroscopic conductance, permeability to Na⁺ and K⁺, stoichiometry of Na⁺ and K⁺ transport at low Na⁺ concentration, and conductance inhibition by K⁺, suggesting that the main functional properties of Ni-OsHKT2;1 were conserved in the G17/V, G17/V D403/E, R21/K R32/K, R21/K R32/K D403/E, F61/S and D403/E variants.

**The OsHKT2;2/1 gene in Nona Bokra**

Amplification by PCR of the 3’ half of the OsHKT2;1 gene from Nona Bokra resulted in a product highly similar to Nipponbare OsHKT2;1 (96.7%), whereas no amplification could be obtained using specific primers for the 5’ half. Analysis of the 3’ amplified fragment and comparison with other sequences of HKT genes showed nucleotide variations that were characteristic of Po-OsHKT2;2, which has only been identified so far in Pokkali. As Nona Bokra like Pokkali is a highly salt-tolerant cultivar, we found it interesting to further investigate the structure of this region. A forward OsHKT2;2 primer (HKT2F) (Figure 3a) and reverse OsHKT2;1 primer (HKT1R) were designed and used to amplify genomic DNA from Nona Bokra. A PCR fragment was obtained and sequenced. The analysis of the sequence revealed a hybrid HKT gene (hereafter named No-OsHKT2;2/f), in which the 5’ half (from translation initiation codon to 1000–1088 bp) was highly similar to the 5’ half of Po-OsHKT2;2, and the 3’ half showed a strong similarity to the second half of Ni-OsHKT2;1. No-OsHKT2;2/1 maintains the Ni-OsHKT2;1 intron structure, containing also two introns of 113 and 292 bp, sharing 100 and 98% identity, respectively, with Ni-OsHKT2;1 introns. To verify the actual expression of the No-OsHKT2;2/1 gene, Nipponbare, Nona Bokra and Pokkali plants were grown under K⁺-starvation conditions, described as inductive conditions for OsHKT2;1 and OsHKT2;2 (Horie et al., 2001), and reverse transcription polymerase chain reaction (RT-PCR) analysis was performed in roots, using different combinations of OsHKT2;1 or OsHKT2;2-specific primers. As expected, we detected the expression of the chimeric OsHKT2;2/1 cDNA in Nona Bokra, of Po-OsHKT2;1 and Po-OsHKT2;2 in Pokkali, and Ni-OsHKT2;1 in Nipponbare (Figure 3a).

Three HKT genes are located near the end of chromosome 6 in rice, all in reverse orientation (Figure 3b). The Nipponbare cultivar contains a truncated OsHKT2;2 isoform, in which the first 263 bp and the last exon at the 3’ end are coincident with the 5’ and 3’ ends of the Po-OsHKT2;2 gene. These two sequences are connected by a 3101-bp fragment without homology to any OsHKT gene. Ni-OsHKT2;1 is located upstream in the chromosome at a distance of 14.9 kb from the truncated Ni-OsHKT2;2, and is preceded by Ni-OsHKT2;4 at 2445 bp. To investigate the OsHKT gene organization in Nona Bokra chromosome 6, a pair of primers was designed based on the sequence upstream from the truncated Ni-OsHKT2;2 in order to amplify the promoter region of No-OsHKT2;2/1. We cloned a 1470-bp fragment upstream from the ATG start codon, which showed 99% identity with the corresponding region of the truncated Ni-OsHKT2;2 genomic sequence and the 5’ end of the Po-OsHKT2;2 cDNA (Figure 4a). A complete OsHKT2;2 gene has only been described in the highly salt-tolerant cultivar Pokkali. Other incomplete OsHKT2;2 fragments can be found in databases, such as that in contig Ctg020871 from the indica reference genome database. The latter contig only possesses the first 164 bp following the ATG codon of an OsHKT2;2 sequence and the upstream region of the gene, displaying 98.84% identity with the upstream region of No-OsHKT2;2/1, excepting a 158-bp insertion.

At the other end of No-OsHKT2;2/1, specific primers recognizing No-OsHKT2;2/1 and Ni-OsHKT2;4 were designed to amplify by PCR a 2600-bp fragment, containing the intermediate region. The amplified fragment has high identity (95%) with the Nipponbare genomic sequence (Figure 4b), showing that in Nona Bokra chromosome 6, No-OsHKT2;2/1 is preceded by No-OsHKT2;4, as is the case
Figure 2. Functional characterization of ectopic variants of OsHKT2;1 expressed in Xenopus oocytes.

(a–h) Comparison of OsHKT2;1 cv Niiponbare (Ni-OsHKT2;1; control transporter) with the triple variant R21/K R32/K D403/E (identified in cv IR58); (a, b, c, d, g) Permeability to K⁺ and Na⁺ (e, f) and (h) Permeability to Na⁺ (c, d, g) and h) Permeability to K⁺. (a–d) Ni-OsHKT2;1 (a, c) and R21/K R32/K D403/E variant (b, d) current-voltage (I-V) relationships in the presence of fixed external K⁺ and varying external Na⁺ (a, b), or fixed external Na⁺ and varying external K⁺ (c, d, g) Inward conductance dependency on external Na⁺ (e) or K⁺ (f) concentrations in the two transporters. Macroscopic inward conductances, extracted from I-V data shown in (a–d) were determined as the slopes of the I-V relationships between the three most negative imposed voltages. (f and h) Sensitivity of Ni-OsHKT2;1 and R21/K R32/K D403/E variant reversal potentials of current (Erev) to external Na⁺ (f) or K⁺ (h) activities. Erev values were obtained from the experiments shown in (a–d). Data in (a–h) are means ± SEs (n = 6).

(i) Comparison of Ni-OsHKT2;1 with the six identified variants (Table S1), G17V (cv GIZA178), G17V/D D403/E (cv Moroberekan), R21/K R32/K (cv Kasalath and LTH), R21/K R32/K D403/E (cv IR58), F61/S (cv Habataki) and D403/E (cv C39). Similar experiments to those presented in (a–h), performed in all variants, were used to define parameters for the comparison of functional properties: Gmax, macroscopic inward conductance at different K⁺ and Na⁺ concentrations (indicated in mV); Gmax 10Na/ Gmax 0.3Na or Gmax 0.3K/Gmax 0.002K; ratio of inward conductances; slope ΔErev, slope of the variation of Erev through OsHKT2;1 transporters with log(Na⁺) activity (slope ΔErev, 0.3–3Na, slope ΔErev, 3–10Na), or with log(K⁺) activity (slope ΔErev 0.002–3 K), when the external Na⁺, or respectively K⁺, concentration varied in the indicated range (in mV). As in (a–h), 0.3 mM K⁺ was present in sets of solutions where Na⁺ varied and 0.3 mM Na⁺ was present in sets of solutions where K⁺ varied. Data are means ± SEs (n = 4–8). Differences between Ni-OsHKT2;1 and variants for all parameters analysed were not significant according to unpaired, two-tailed Student’s t-tests (P < 0.05).

<table>
<thead>
<tr>
<th>OsHKT2;1 variants</th>
<th>Gmax 0.3K 0.3Na (µS)</th>
<th>Gmax 10Na/Gmax 0.3Na</th>
<th>slope ΔErev 0.3 to 3 Na (mV/decade)</th>
<th>slope ΔErev 3 to 9 Na (mV/decade)</th>
<th>Gmax 0.3K/Gmax 0.002K</th>
<th>slope ΔErev 0.002 to 3 K (mV/decade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni-OsHKT2;1</td>
<td>38 ± 2</td>
<td>2.0 ± 0.2</td>
<td>21 ± 5</td>
<td>41 ± 6</td>
<td>0.51 ± 0.06</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>G17V</td>
<td>44 ± 3</td>
<td>2.1 ± 0.2</td>
<td>27 ± 5</td>
<td>41 ± 2</td>
<td>0.50 ± 0.01</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>G17V D403/E</td>
<td>36 ± 1</td>
<td>1.8 ± 0.1</td>
<td>14 ± 2</td>
<td>41 ± 4</td>
<td>0.48 ± 0.02</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>R21/K R32/K</td>
<td>38 ± 2</td>
<td>1.8 ± 0.1</td>
<td>18 ± 3</td>
<td>40 ± 5</td>
<td>0.51 ± 0.03</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>R21/K R32/K D403/E</td>
<td>40 ± 2</td>
<td>1.9 ± 0.1</td>
<td>18 ± 2</td>
<td>39 ± 5</td>
<td>0.53 ± 0.03</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>F61/S</td>
<td>39 ± 6</td>
<td>1.9 ± 0.1</td>
<td>24 ± 3</td>
<td>48 ± 2</td>
<td>0.48 ± 0.02</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>D403/E</td>
<td>38 ± 1</td>
<td>2.0 ± 0.2</td>
<td>17 ± 2</td>
<td>38 ± 4</td>
<td>0.54 ± 0.01</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>
in Nipponbare (Figure 3b). This whole set of data indicated a reorganization in Nona Bokra chromosome 6 by a genomic deletion, producing a chimera between the 5’ end of No-OsHKT2;2 and the 3’ end of No-OsHKT2;1. It is worth noting that No-OsHKT2;2/1 possesses, like OsHKT2;2, and in contrast to OsHKT2;1, a conserved glycine residue in its four pore-loop regions, which is considered to be an important determinant of K⁺ permeability in HKT transporters (Mäser et al., 2002) (Figure 4c).

Expression of No-OsHKT2;2/1 is regulated by K⁺ and Na⁺ concentrations

It has been reported that the expression of OsHKT2;1 is strongly affected by external concentrations of Na⁺ and K⁺ (Garcia-deblás et al., 2003). We have therefore performed quantitative RT-PCR analysis for Ni- and Po-OsHKT2;1, Po-OsHKT2;2 and No-OsHKT2;2/1 in roots of plants grown for 14 days in the presence of different ion concentrations (Figure 5a). The three HKT genes showed similar expression patterns, with the highest level of expression under K⁺-free conditions. When plants were grown in the presence of 30 mM K⁺, expression decreased to nearly undetectable levels. The presence of high Na⁺ (30 mM) levels also reduced expression levels for the three OsHKTs. However, compared with the effect of high K⁺, this reduction was much less severe, and a significant level of expression was maintained.

We have also examined the effect of sudden salt stress on the expression of Ni- and Po-OsHKT2;1, Po-OsHKT2;2 and No-OsHKT2;2/1. Plants were grown for 21 days in hydroponics, and were then subsequently treated with 30 mM NaCl (Figure 5b). Root and leaf expression levels were determined before and 1, 5, 24, 48 and 72 h after NaCl treatment. Interestingly, both Po-OsHKT2;2 and No-OsHKT2;2/1 were hardly detectable in leaves, in contrast to the OsHKT2;1 gene from Nipponbare or Pokkali. In leaves, the expression level of OsHKT2;1 in Nipponbare and Pokkali transiently increased after the stress application, then decreased to levels similar to those before the NaCl treatment. In roots, the response of the four genes was similar, and was characterized by an initial reduction in expression level during the first hours of treatment, followed by a transient restoration (complete in OsHKT2;1) then stabilization (in No-OsHKT2;2/1), or again a decay to levels below those before the addition of NaCl. Altogether, these results suggest that No-OsHKT2;2/1 could play a role in roots in conditions of low K⁺ availability as well as salt stress. Similar results were obtained when plants were treated with 100 mM NaCl, except that Ni- and Po-OsHKT2;1 expression in leaves displayed a transient reduction before the peak in transcription levels after the stress application (Figure S1).

No-OsHKT2;2/1 functions as an Na⁺–K⁺ symporter, even at high external Na⁺ concentrations

The protein sequence comparison between the three rice HKTs showed that No-OsHKT2;2/1 is a chimera in which N- and C-termini are highly similar to those of Po-OsHKT2;2 and Ni-OsHKT2;1, respectively: the first three MPM domains are homologous with OsHKT2;2, and the last MPM domain is homologous with OsHKT2;1. The function of No-OsHKT2;2/1 was investigated by heterologous expression in yeast cells and Xenopus oocytes. Parallel experiments were carried out with Ni-OsHKT2;1 and Po-OsHKT2;2, for a fine comparison.

For yeast expression, the full-length cDNAs were cloned in the pYPGE15 yeast expression vector under the PGK1 constitutive expression promoter, and transformed into S. cerevisiae strain W306, which is deficient in its endogenous K⁺ uptake systems TRK1 and TRK2. This strain cannot
Figure 4. Comparison of OsHKT2;1 and OsHKT2;2 sequences from different cultivars.
(a, b) Sequence alignments of the 5' region (a) and 3' region (b) of Nona Bokra No-OsHKT2;2/1, Nickponbare Ni-OsHKT2;1 and truncated Ni-OsHKT2;2, Pokkali Po-OsHKT2;1 and Po-OsHKT2;2, and region of contig02081 from the reference genome with homology to OsHKT2;2.
(c) Amino acid sequence alignment of the four putative selective pore-forming regions (P1–P4). Arrowheads indicate the amino acid position where a glycine residue is conserved in Na⁺–K⁺ symporters. Residues that differ from OsHKT2;1 are shown in grey.

Growth at low K⁺ concentrations because it does not take up K⁺ or Na⁺ at low concentrations. Serial dilution growth tests with the three clones revealed that both No-OsHKT2;2/1 and Po-OsHKT2;2 cDNAs restored the defective growth of the yeast mutant strain at low K⁺, whereas the Ni-OsHKT2;1 was ineffective (Figure 6a). In K⁺ and Na⁺ uptake experiments at low concentrations (50 μM), yeast transformed with No-OsHKT2;2/1 and Po-OsHKT2;2 clones transported both K⁺ and Na⁺ when the two ions were added independently, but the uptake rate of both cations increased when they were added together (Figure 6b,c,d). Consistent with the growth experiments, Ni-OsHKT2;1 transformed cells took up Na⁺ very efficiently, but failed to take up K⁺. These results indicate that both No-OsHKT2;2/1 and Po-OsHKT2;2 functioned as Na⁺–K⁺ symporters when expressed in yeast, whereas Ni-OsHKT2;1 behaved essentially as an Na⁺ transporter.

When No-OsHKT2;2/1 was expressed in Xenopus oocytes, functional data were in agreement with those obtained upon expression in yeast. At low external K⁺ and Na⁺ concentrations, No-OsHKT2;2/1 was found to conduct both Na⁺ and K⁺, as attested by positive shifts of I–V relationships along the voltage axis when the concentration of one of the two ions was fixed and that of the other one increased (Figure 7a,b,d,n). Slopes of variation of No-OsHKT2;2/1 Erev with Na⁺ or K⁺ activity close to 20 mV per activity decade for both ions (Figure 7m,n) indicated that Na⁺ and K⁺ were the main ions transported through No-OsHKT2;2/1, and that their transport stoichiometry was, as in Ni-OsHKT2;1, close to 1:1 (Jabnoune et al., 2009). Comparing No-OsHKT2;2/1 in Xenopus oocytes with Po-OsHKT2;2 and Ni-OsHKT2;1, No-OsHKT2;2/1 appeared to behave more similarly to Po-OsHKT2;2 than to Ni-OsHKT2;1, as observed when expressed in yeast (Figure 6). Indeed, the three transporters displayed permeability to both Na⁺ and K⁺ at low external K⁺ and Na⁺ concentrations when expressed in oocytes (Figure 7a,b,d,e,g,h), but displayed strong differences in their conductance sensitivity to K⁺ (Figure 7a,d,g,j). In No-OsHKT2;2/1 and Po-OsHKT2;2, increasing external K⁺ up to 1 mM in the presence of 0.3 mM Na⁺ increased the transporter inward conductance, an inhibitory effect of external K⁺ on the conductance being observed only when the K⁺ concentration was raised above 3 mM. In Ni-OsHKT2;1, the K⁺ threshold producing inhibition of the transporter conductance was more than 10 times lower. Thus, except at very low external K⁺ concentration (≤100 μM), the
conductances of No-OsHKT2;2/1 and Po-OsHKT2;2 were higher than that of Ni-OsHKT2;1 (e.g. four and eight times higher, respectively, in the presence of 1 mM K⁺; Figure 7j).

At high external Na⁺ concentration, it has been reported that Na⁺−K⁺ symports of the HKT family lose their ability to transport K⁺ (Gassmann et al., 1996; Jaboue et al., 2009). To further study the behaviour of No-OsHKT2;2/1, Po-OsHKT2;2 and Ni-OsHKT2;1 at high Na⁺ concentrations, we tested the growth of yeast mutant cells (strain W48), expressing these transporters, at 50 μM K⁺ in the presence of 100 mM Na⁺ (Figure 6a). Both No-OsHKT2;2/1 and Po-OsHKT2;2 restored the defective growth of the yeast mutant at this low K⁺ in the presence of 100 mM Na⁺ (Figure 6a), which indicated that they still transport K⁺ in the presence of high Na⁺ concentration.

Similar conclusions could be drawn from Xenopus oocyte experiments in the presence of 30 mM Na⁺ and varying K⁺ concentrations. No-OsHKT2;2/1 and Po-OsHKT2;2 still appeared to be permeable to K⁺ under these conditions (Figure 7c,i), in contrast to Ni-OsHKT2;1 which was then in Na⁺ uniport mode (Figure 7f; Jaboue et al., 2009). Indeed, shifts of i-V relationships along the voltage axis upon increased K⁺ concentration in the presence of 30 mM Na⁺ were observed in No-OsHKT2;2/1 and Po-OsHKT2;2, but not in Ni-OsHKT2;1 (Figure 7c,f,i,o). In the presence of 30 mM Na⁺ alone (nominal K⁺ concentration), the inward conductance of the three transporters was similar (Figure 7i). When K⁺ was also present at a concentration ≥1 mM, the conductances of No-OsHKT2;2/1 and Po-OsHKT2;2 were strongly increased, in agreement with Na⁺−K⁺ symport behaviour, whereas that of Ni-OsHKT2;1 was inhibited (Figure 7i). Thus, the inward conductances of No-OsHKT2;2/1 and Po-OsHKT2;2, in the millimolar range, were much higher than that of Ni-OsHKT2;1 (between six and 30 times higher) at high external Na⁺ and K⁺ concentrations. It should be noted that in No-OsHKT2;2/1 and Po-OsHKT2;2, in contrast to what was observed at low Na⁺ concentration, increasing external K⁺ concentration up to 10 mM had no (in Po-OsHKT2;2) or very limited (in No-OsHKT2;2/1) inhibitory effect on the inward conductance of the transporters.

Finally, these results obtained in yeast and in Xenopus oocytes show that the Nona Bokra No-OsHKT2;2/1 transporter works as an Na⁺−K⁺ symporter in a wide range of Na⁺ and K⁺ concentrations. They suggest in particular that it is a very efficient system for mediating high-affinity K⁺ uptake, even in conditions of high Na⁺ concentrations. No-OsHKT2;2/1 behaves very similarly to Po-OsHKT2;2, and in sharp contrast to Ni-OsHKT2;1, which displays limited (in oocyte) or no (in yeast) substantial permeability to K⁺.
Comparison with the homologous sequence in the salt-sensitive Koshihikari cultivar, leading to four amino acid changes. The Nona Bokra OsHKT1;5 transporter displayed a higher Na⁺ transport activity, likely explaining the salt tolerance QTL associated with the Nona Bokra OsHKT1;5 allele. Another example concerns Arabidopsis populations, originating from coastal and saline soils, in which the presence of a weak AtHKT1;1 allele, resulting from polymorphism in the promoter region, was shown to affect salt tolerance (Rus et al., 2006; Baxter et al., 2010). Our SNP analysis focused on the coding sequence of OsHKT2;1 and revealed nine nucleotide changes, of which only five were non-synonymous. The different residue substitutions were distributed in the N-terminal cytosolic region and at the end of the first and sixth transmembrane segments, i.e. in transporter regions whose possible role in determining transport characteristics had not been previously examined (Corratgé-Faille et al., 2010). We have characterized all identified variants by expression in Xenopus oocytes and found no significant difference in OsHKT2;1 ion selectivity or conductivity (Figure 2). The observed degree of conservation of the genomic sequence as well as the unchanged transport characteristics suggests the importance of authentic HKT2;1 activity in the plant. This hypothesis is also supported by the strong conservation of the OsHKT2;1 promoter sequence between the different varieties. Only 13 SNPs have so far been reported in the putative promoter sequence, up to 5 kb upstream from the coding sequence (http://oryzasnp.plantbiology.msu.edu). None of these SNPs seems to affect putative regulatory motifs involved in salt or drought stress (cf. plant cis-acting regulatory DNA elements in the PLACE database, http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999). Interestingly, we found that the salt-tolerant Nona Bokra cultivar does not possess an OsHKT2;1 gene (Figure 3), but instead it has a chimeric gene containing only part of OsHKT2;1.

**Identification of a new HKT gene in a salt-tolerant cultivar.** Our SNP analysis has led to the identification of a new OsHKT isoform, No-OsHKT2;2/1, in Nona Bokra. The relative high salt tolerance of this cultivar makes the identification of this chimeric gene particularly interesting from an evolutionary point of view. The HKT gene family in rice comprises nine genes (García-de-la-Bañas et al., 2003), corresponding to five members of subfamily 1 and four members of subfamily 2, distributed on chromosomes 1, 2, 4 and 6. The genome of *japonica* cv Nipponbare contains seven functional genes and two pseudogenes, OsHKT1;2 with three stop codons in its mRNA and OsHKT2;2 interrupted by a 3.1-kb DNA fragment. OsHKT2;2 is located on chromosome 6, and shares a high similarity at the sequence level, but not at the functional level, with OsHKT2;1 (Horie et al., 2001), which is located 14.9-kb upstream. So far a complete OsHKT2;2 gene has only been described in Pokkali, which is a salt-tolerant indica.

**Discussion**

**Analysis of DNA polymorphism in different rice cultivars shows a high conservation of the OsHKT2;1 gene**

Analyses of genetic polymorphism can help to unravel adaptation mechanisms. In the salt-tolerant Nona Bokra cultivar, Ren et al. (2005) identified six SNPs in OsHKT1;5 in comparison with the homologous sequence in the salt-sensitive Koshihikari cultivar, leading to four amino acid changes. The Nona Bokra OsHKT1;5 transporter displayed a higher Na⁺ transport activity, likely explaining the salt tolerance QTL associated with the Nona Bokra OsHKT1;5 allele. Another example concerns Arabidopsis populations, originating from coastal and saline soils, in which the presence of a weak AtHKT1;1 allele, resulting from polymorphism in the promoter region, was shown to affect salt tolerance (Rus et al., 2006; Baxter et al., 2010). Our SNP analysis focused on the coding sequence of OsHKT2;1 and revealed nine nucleotide changes, of which only five were non-synonymous. The different residue substitutions were distributed in the N-terminal cytosolic region and at the end of the first and sixth transmembrane segments, i.e. in transporter regions whose possible role in determining transport characteristics had not been previously examined (Corratgé-Faille et al., 2010). We have characterized all identified variants by expression in Xenopus oocytes and found no significant difference in OsHKT2;1 ion selectivity or conductivity (Figure 2). The observed degree of conservation of the genomic sequence as well as the unchanged transport characteristics suggests the importance of authentic HKT2;1 activity in the plant. This hypothesis is also supported by the strong conservation of the OsHKT2;1 promoter sequence between the different varieties. Only 13 SNPs have so far been reported in the putative promoter sequence, up to 5 kb upstream from the coding sequence (http://oryzasnp.plantbiology.msu.edu). None of these SNPs seems to affect putative regulatory motifs involved in salt or drought stress (cf. plant cis-acting regulatory DNA elements in the PLACE database, http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999). Interestingly, we found that the salt-tolerant Nona Bokra cultivar does not possess an OsHKT2;1 gene (Figure 3), but instead it has a chimeric gene containing only part of OsHKT2;1.

**Identification of a new HKT gene in a salt-tolerant cultivar.** Our SNP analysis has led to the identification of a new OsHKT isoform, No-OsHKT2;2/1, in Nona Bokra. The relative high salt tolerance of this cultivar makes the identification of this chimeric gene particularly interesting from an evolutionary point of view. The HKT gene family in rice comprises nine genes (García-de-la-Bañas et al., 2003), corresponding to five members of subfamily 1 and four members of subfamily 2, distributed on chromosomes 1, 2, 4 and 6. The genome of *japonica* cv Nipponbare contains seven functional genes and two pseudogenes, OsHKT1;2 with three stop codons in its mRNA and OsHKT2;2 interrupted by a 3.1-kb DNA fragment. OsHKT2;2 is located on chromosome 6, and shares a high similarity at the sequence level, but not at the functional level, with OsHKT2;1 (Horie et al., 2001), which is located 14.9-kb upstream. So far a complete OsHKT2;2 gene has only been described in Pokkali, which is a salt-tolerant indica.

**Discussion**

**Analysis of DNA polymorphism in different rice cultivars shows a high conservation of the OsHKT2;1 gene**

Analyses of genetic polymorphism can help to unravel adaptation mechanisms. In the salt-tolerant Nona Bokra cultivar, Ren et al. (2005) identified six SNPs in OsHKT1;5 in comparison with the homologous sequence in the salt-sensitive Koshihikari cultivar, leading to four amino acid changes. The Nona Bokra OsHKT1;5 transporter displayed a higher Na⁺ transport activity, likely explaining the salt tolerance QTL associated with the Nona Bokra OsHKT1;5 allele. Another example concerns Arabidopsis populations, originating from coastal and saline soils, in which the presence of a weak AtHKT1;1 allele, resulting from polymorphism in the promoter region, was shown to affect salt tolerance (Rus et al., 2006; Baxter et al., 2010). Our SNP analysis focused on the coding sequence of OsHKT2;1 and revealed nine nucleotide changes, of which only five were non-synonymous. The different residue substitutions were distributed in the N-terminal cytosolic region and at the end of the first and sixth transmembrane segments, i.e. in transporter regions whose possible role in determining transport characteristics had not been previously examined (Corratgé-Faille et al., 2010). We have characterized all identified variants by expression in Xenopus oocytes and found no significant difference in OsHKT2;1 ion selectivity or conductivity (Figure 2). The observed degree of conservation of the genomic sequence as well as the unchanged transport characteristics suggests the importance of authentic HKT2;1 activity in the plant. This hypothesis is also supported by the strong conservation of the OsHKT2;1 promoter sequence between the different varieties. Only 13 SNPs have so far been reported in the putative promoter sequence, up to 5 kb upstream from the coding sequence (http://oryzasnp.plantbiology.msu.edu). None of these SNPs seems to affect putative regulatory motifs involved in salt or drought stress (cf. plant cis-acting regulatory DNA elements in the PLACE database, http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999). Interestingly, we found that the salt-tolerant Nona Bokra cultivar does not possess an OsHKT2;1 gene (Figure 3), but instead it has a chimeric gene containing only part of OsHKT2;1.

**Identification of a new HKT gene in a salt-tolerant cultivar.** Our SNP analysis has led to the identification of a new OsHKT isoform, No-OsHKT2;2/1, in Nona Bokra. The relative high salt tolerance of this cultivar makes the identification of this chimeric gene particularly interesting from an evolutionary point of view. The HKT gene family in rice comprises nine genes (García-de-la-Bañas et al., 2003), corresponding to five members of subfamily 1 and four members of subfamily 2, distributed on chromosomes 1, 2, 4 and 6. The genome of *japonica* cv Nipponbare contains seven functional genes and two pseudogenes, OsHKT1;2 with three stop codons in its mRNA and OsHKT2;2 interrupted by a 3.1-kb DNA fragment. OsHKT2;2 is located on chromosome 6, and shares a high similarity at the sequence level, but not at the functional level, with OsHKT2;1 (Horie et al., 2001), which is located 14.9-kb upstream. So far a complete OsHKT2;2 gene has only been described in Pokkali, which is a salt-tolerant indica.
Figure 7. Comparison of functional properties of No-OsHKT2;2/1, Ni-OsHKT2;1 and Po-OsHKT2:2 expressed in Xenopus oocytes. (a, d, g and m) Permeability to K⁺ at low (0.3 mM) external Na⁺. (b, e, h and n) Permeability to Na⁺ at low (0.1 mM) external K⁺. (c, f, i and o) Permeability to K⁺ at high (30 mM) external Na⁺. (a–i) Current-voltage (I–V) relationships of No-OsHKT2;2/1 (a–c), Ni-OsHKT2;1 (d–f) and Po-OsHKT2:2 (g–i). Data are means ± SEs (n = 3). (j, k and l) Comparison of inward conductance (determined as in Figure 2) dependency on K⁺ (j, low Na⁺), Na⁺ (k, high Na⁺) or Na⁺ (l) in the three transporters. (m, n and o) Variation of the reversal potential of current (Eᵣ) through the three transporters with K⁺ (m, low Na⁺), Na⁺ (n) or Na⁺ (o) activities. Data in (j–o) are means ± SEs (n = 3) (same experiments as in panels a–i).
cultivar. Database searches in other rice cultivars only showed contigs with partial 5' end sequences of OsHKT2;2 (as Ctg020871 from the indica reference genome). Sequence analysis of the genomic region flanking the here identified No-OsHKT2;2/1 gene revealed a deletion of approximately 15 kb that caused a chimeric gene by fusing the 5' OsHKT2;2 region (about three-quarters of the coding sequence) with the 3' OsHKT2;1 region (about one-quarter of the coding sequence). Based on the conservation of the OsHKT2;1 intron structure in No-OsHKT2;2/1 and a comparison of the coding sequences, the junction between OsHKT2;2 and OsHKT2;1 is likely to be located within the first exon. Altogether these data suggest that the chromosomal area between OsHKT2;1 and OsHKT2;2 is susceptible to reorganization events. The reorganization found in Nona Bokra (Figure 3) has led to a 15-kb deletion, resulting in the chimeric gene No-OsHKT2;2/1. Finally, it should be noted that the only cultivars in which a functional OsHKT2;2 gene or OsHKT2;2 'variant' have been identified so far are Pokkali (Horie et al., 2001) and Nona Bokra (Figure 3). Both are considered as salt tolerant, which may indicate that the presence of OsHKT2;2 constitutes an evolutionary advantage for these two cultivars.

**Physiological role of No-OsHKT2;2/1**

In order to investigate the role of the newly identified No-OsHKT2;2/1 gene in Nona Bokra, we have compared its expression pattern and its functional characteristics with those of OsHKT2;1 and OsHKT2;2 from Nipponbare and/or Pokkali. The expression levels in Nona Bokra and Pokkali plants grown at different Na+ and K+ concentrations showed that the four HKTs are upregulated in roots upon K+ shortage (Figure 5a). Differences between OsHKT2;1, in Nipponbare or Pokkali, and No-OsHKT2;2/1 and Po-OsHKT2;2 were also observed. Whereas OsHKT2;1 showed comparable expression levels in leaves and roots, OsHKT2;2/1 and OsHKT2;2 were only expressed in roots. In response to NaCl stress, root expression levels of the four genes were found to be downregulated, although not abolished. When analysed over time, after 72 h of NaCl treatment, the downregulation was less pronounced for No-OsHKT2;2/1, which may reflect a more important role of No-OsHKT2;2/1 in roots of plants subjected to high Na+. The functional characterization of No-OsHKT2;2/1, Ni-OsHKT2;1 and Po-OsHKT2;2 has also revealed interesting differences. The results obtained for the permeability to Na+ and K+ of the three HKT transporters in Xenopus oocytes and S. cerevisiae are consistent. As described previously, Ni-OsHKT2;1 was found to behave as a high-affinity Na+ transporter, except at low K+ and Na+ concentrations, where it functions as an Na+-K+ symport when expressed in Xenopus oocytes (Figures 6 and 7; Horie et al., 2001; Golliday et al., 2002; Jabnoun et al., 2009). Po-OsHKT2;2, on the other hand, is highly permeable to both K+ and Na+ (Horie et al., 2001; Yao et al., 2010) in a large range of concentrations, and functions preferentially as a symport (Figures 6 and 7). The transport characteristics of the chimeric No-OsHKT2;2/1 are very similar to those of Po-OsHKT2;2. A small difference concerned a slightly stronger inhibition by external K+ in No-OsHKT2;2/1. No-OsHKT2;2/1 and Po-OsHKT2;2 can thus both be expected to co-transport Na+ and K+ in planta in the presence of varying Na+ and K+ concentrations, in contrast to OsHKT2;1.

The high expression levels of the three HKTs in plants grown at low concentrations of K+ suggest that these transporters play a role in conditions of K+ starvation. Ni-OsHKT2;1 has indeed been shown to play an important role in nutritional Na+ uptake by roots under K+ starvation (Horie et al., 2007). K+ transport through HKT transporters has not yet been evidenced in planta (Laurie et al., 2002, Haro et al., 2006). However, upon overexpression in cultured tobacco cells, OsHKT2;2 was shown to co-transport Na+ and K+ (Yao et al., 2010). Considering the high expression of No-OsHKT2;2/1 in roots in low K+ conditions and the high permeability to K+ observed in yeast and oocytes, we could hypothesize that No-OsHKT2;2/1 in Nona Bokra co-transport Na+ and K+ in roots. Also, the relatively maintained expression of No-OsHKT2;2/1 in plants grown at 30 mM Na+, and the recovery of expression 72 h after high salt treatments, also suggests a role of this transporter during salt stress. Both the Xenopus oocyte and yeast experiments show that No-OsHKT2;2/1 maintains a K+ permeability at high external Na+ concentrations, even at very low K+ concentrations (Figures 6 and 7). We can thus propose that No-OsHKT2;2/1 is still involved in Na+ and K+ co-transport in planta under high salt concentrations, thereby facilitating the uptake of K+ and thus contributing to maintaining an acceptable K+ /Na+ ratio in saline conditions. It can therefore be hypothesized that by playing a role in K+ nutrition of Nona Bokra plants, even in saline conditions, No-OsHKT2;2/1 contributes to the salt tolerance of the Nona Bokra cultivar.

**EXPERIMENTAL PROCEDURES**

**Plant material and growth conditions**

The rice core collection was obtained from different germplasm collections: the International Rice Research Institute (IRRI, http://irri.org/), US National Plant Germplasm System (NPGS, http://www.ars-grin.gov/npgs/), Rice Genome Resource Center (RGRC, http://www.rgrc.dna.afrc.go.jp/), Instituto Valenciano de Investigaciones Agrarias (IVIA, http://www.ivia.es/) and the Pohang University of Science and Technology (POSTECH, http://www.postech.ac.kr/). Seeds from different cultivars were germinated and grown in soil in growth chambers at 27°C on a 12-h light (170 µmol m⁻² s⁻¹)/12-h dark schedule. Fifteen days after germination DNA was extracted from leaf tissue.

For expression analysis in varying Na+ and K+ conditions, hydroponic cultures were performed on Yoshida medium (Yoshida et al., 1976), in which K+ salts were substituted to various degrees with NH₄⁺ or Na+, and brought to pH 5.5 with Tris-HCl. Seeds were germinated...
on plastic mesh in modified media before transfer to the hydropionic cultures at 27°C on a 12-h light/12-h dark schedule. For NaCl treatments, pre-germinated seeds were sown at two seeds per hole on a Styrofoam sheet with a nylon net bottom. The sheets were floated on a plastic tray filled with distilled water for 3 days, and then in nutrient solution at pH 5.5 for 3 days (27°C and 70% relative humidity). After 18 days, the salinity of the culture media was increased by adding 30 or 100 mM NaCl. Samples were collected and frozen at 0, 1, 5, 24, 48 and 72 h, and RNA was isolated.

**EcoTilling analysis**

To detect allelic polymorphisms present in each cultivar, seeds were germinated and leaf tissue from 15-day-old seedlings was frozen and DNA extracted, using a DNeasy plant mini kit (Qiagen, http://www.qiagen.com). Specific primers were designed to amplify the OsHKT2;1 (forward, HKT1F, 5'-CATACCTGGTGCTCGTGGC-3', and reverse, HKT1R, 5'-ACTATGCGCCAAATTACGAAA CCTGA-3'). DNA from each cultivar was amplified and the individual PCR products were visualized on 1.5% or Nipponkayó® PCR amplification products in equal quantities and, after densitometry and re-naturing to allow the formation of heteroduplex DNA molecules, they were digested with endonuclease CLEI, I isolated from celery. Fragments were separated and analysed by electrophoresis using agarose gels. Cultivars were grouped according to the electrophoretic patterns observed, and DNA fragments from two or three cultivars representing each group were sequenced to determine the polymorphism.

**RNA isolation, semi-quantitative reverse-transcriptase-PCR and quantitative real-time PCR assays**

Total RNA was isolated with the RNeasy plant mini kit (Qiagen), followed by DNase digestion. The RNA concentration was determined by a fluorometric assay with the kit Quant-IT™ Ribogreen® RNA Assay Kit (Molecular Probes Inc., http://www.invitrogen.com), according to the manufacturer's instructions. Synthesized cDNAs were used for PCR amplification. For semi-quantitative RT-PCR analysis, specific primers were used for OsHKT2;1 (HKT1F and HKT1R) and OsHKT2;2 transcripts (forward, HKT2F, 5'-TCA TAGTCTCTGCTGGTAAA-3' and reverse, HKT2R, 5'-TCTACGATT CAAAAGGGCCCTAA-3'). The amplification of a fragment of the ACT71 gene was used as a standard control (forward, 5'-CCGCTATGG TAGTGGCCCA-3', reverse, 5'-CTGAGAGATCGGAAATG-3').

One-step real-time PCRs were performed as previously described (Domingo et al., 2009) on a LightCycler® 2.0 (Roche Applied Science http://www.roche-applied-science.com/index.jsp, GE Healthcare, http://www.gehealthcare.com), using the LightCycler® Fast Start DNA MasterPlus Sybr Green I kit (Roche Applied Science, GE Healthcare). Total RNA (100 ng) was used in each assay. Primers were used for Ni- and Po-OsHKT2;1 (forward, HKT1F and reverse, HKT1R, 5'-GACAGAGACCTCCCTTACGAGG-3'), Po-OeHKT2;2 and No-OsHKT2;2/1 transcripts (forward, HKT2R, and reverse, HKT2H). The RT-PCR procedure consisted of an incubation at 48°C for 20 min, followed by 45 cycles at 95°C for 2 s, 58°C for 8 s and 72°C for 13 s. The values presented are the mean of two biological replicates, each with two technical replicates. The error bars indicate the standard deviation from the mean.

**Expression of rice HKT transporters in Xenopus laevis oocytes**

The Ni-OsHKT2;1, Po-OsHKT2;2 and No-OsHKT2;2/1 cDNAs were subcloned into the pGEMGD vector (D. Becker, University of Würzburg, Germany) between the 5' and 3' untranslated regions of the Xenopus β-globin gene. The cDNAs of the different ecotypic variants of OsHKT2;1 were obtained from Ni-OsHKT2;1 cDNA by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The following pairs of forward (F) and reverse (R) primers, introducing a simple base pair mutation (underlined), were used for mutagenesis: G3'-5'-CACTTCAGAGGGGTTCTCGAG-3' and reverse, HKT1R, 5'-ACTATGCGCCAAATTACGAAA CCTGA-3'. DNA from each cultivar was amplified and the individual PCR products were visualized on 1.5% or Nipponkayó® PCR amplification products in equal quantities and, after densitometry and re-naturing to allow the formation of heteroduplex DNA molecules, they were digested with endonuclease CLEI, I isolated from celery. Fragments were separated and analysed by electrophoresis using agarose gels. Cultivars were grouped according to the electrophoretic patterns observed, and DNA fragments from two or three cultivars representing each group were sequenced to determine the polymorphism.

**Expression of HKT transporters in yeast**

The S. cerevisiae strain W3A (Mat a ade2 ura3 trp1 trk1::LEU2 trk2::HIS3), which is deficient in its endogenous K⁺ uptake systems, TRK1 and TRK2 (Haro and Rodríguez-Navarro, 2003), was used for expressing the HKT transporters. The yeast strains were normally grown either in the complex medium YPD (1% yeast extract, 2% peptone, 2% glucose) or in the minimal SD medium (yeast nitrogen base 0.67%, (NH₄)₂SO₄, 0.5%, glucose 2%; Sherman, 1991; ), supplemented with 50 mM K⁺ and the appropriate auxotrophic factors. For functional expression tests in yeast cells, the Ni-OsHKT2;1, Po-OsHKT2;2 and No-OsHKT2;2/1 cDNAs were subcloned into pYPG15 (Brunelli and Pall, 1993), in which expression is under the control of the PGK1 constitutive expression promoter.
Growth tests of *S. cerevisiae* transformants were performed by the drop test method on arginine phosphate (AP) medium (Rodríguez-Navarro and Ramos, 1984), supplemented with KCl or NaCl at the indicated K⁺ and Na⁺ concentrations.

The Na⁺ and K⁺ uptake experiments were performed in K⁺-starved cells, as previously described (Rodríguez-Navarro and Ramos, 1984; Bañuelos et al., 2002). First, cells were grown overnight in AP medium supplemented with 50 mm K⁺, washed with water and resuspended in K⁺and Na⁺-free AP medium. The starvation time was 4 h. Uptake tests were performed in 10 mM MES- Ca²⁺, pH 6.0, supplemented with 2% glucose (testing buffer). The experiments were started by the addition of the selected cation. At regular intervals, samples were taken and centrifuged at 1000 g for 1 min and the external concentration of the tested cation was determined in the supernatant by atomic emission spectrophotometry. Experiments were repeated four times.

ACKNOWLEDGEMENTS

This work was supported by the European Research Area Network Plant Genomics Programme (grant no. ERA-PG FP/06.018B to HS, AR-N and MT), the Biotechnology and Biological Sciences Research Council-Institut National de la Recherche Agronomique (grant to HS and A-VA) and the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) (financial support and grant no. RTA2010-00048-00-00 to CD).

SUPPORTING INFORMATION

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

**Figure S1.** Variation of expression levels of No-OsHKT2;2/1, Ni-OsHKT2;1, Po-OsHKT2;1 and Po-OsHKT2;2 in rice plants subjected to 100 mM NaCl.

**Table S1.** Detailed list of variations in the OsHKT2;1 coding sequence found in the cultivars analysed, and the resulting amino acids.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES


Mäser, P., Hoseoo, Y., Goshima, S. et al. (2002) Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-


