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Review

Functions of HKT transporters in sodium transport in roots and in protecting leaves from salinity stress

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Abstract Excessive sodium (Na^+) accumulation in plants due to soil salinity is toxic to most higher plants including crop plants. Many genes encoding Na^+ permeable transporters/channels have been identified for the last 15 years, based on genetic approaches, genome-sequencing projects and functional complementation screening using yeast mutants. The HKT-type transporter/channel class is one of the best characterized Na^+ permeable membrane proteins in plants. Interestingly, most Na^+ permeable proteins including HKT-type transporters/channels in plants were shown or deduced to play a protective role against salinity stress. A Na^+ selective transporter/channel in rice (*Oryza sativa*), OsHKT2;1, however, has recently been proven to function in "nutritional Na^+ absorption" in K^+-starved roots rather than functioning in a protective role under salinity stress. Here we review findings on the HKT-type transporters/channels, mainly focusing on the function of OsHKT2;1 that is tightly regulated by K^+/Na^+ homeostatic mechanisms of rice plants. We also discuss functions of *Arabidopsis thaliana* AtHKT1;1 and rice OsHKT1;5 in protecting plant leaves from over-accumulating toxic Na^+ concentrations during salinity stress by removing Na^+ from the xylem sap.

Key words: K^+/Na^+ transporter, *Oryza sativa*, *Arabidopsis thaliana*, P-loop domain, nutritional Na^+ uptake.

K^+ is the most abundant cation in plant cells and plays essential roles in osmotic regulation, cell growth, enzyme activity, membrane polarization and salt tolerance. Plants have many kinds of K^+ transporters and some of them function in the uptake of K^+ from the soil to cells across plasma membranes (Hirsch et al. 1998; Gierth et al. 2005). A number of cDNAs encoding plant K^+ channels and transporters have been isolated and characterized (Sentenac et al. 1992; Anderson et al. 1992; Schachtman and Schroeder, 1994; Santa-Maria et al. 1997; Kim et al. 1998; Fu and Luan, 1998; Fairbairn et al. 2000). Analyses of the TaHKT2;1 (previously named TaHKT1) from wheat (*Triticum aestivum*) (Platten et al. 2006) were the first reports for the HKT-type transporter/channel in plants (Schachtman and Schroeder 1994; Rubio et al. 1995). TaHKT2;1 has been shown to mediate high affinity Na^+/K^+ co-transport and low affinity Na^+ transport at high Na^+ concentrations in yeast cells and *Xenopus laevis* oocytes (Rubio et al. 1995; Gassmann et al. 1996). TaHKT2;1 mediates high rates of Na^+ influx at low and high Na^+ concentrations (Rubio et al. 1995; Gassmann et al. 1996). These studies indicate a channel-like transport mode of TaHKT2;1 (Gassmann et al. 1996). Subsequently, many cDNA encoding proteins belonging to the HKT family have been cloned from various plants and functional analysis of each HKT is advanced. We summarize here functions of HKT proteins in the K^+ and Na^+ transport systems in rice (*Oryza sativa*) and *Arabidopsis thaliana*.

Identification of *OsHKT2;1* and *OsHKT2;2* cDNA, and their ion selectivity in heterologous expression systems

Complementary DNAs encoding the HKT-type transporter/channel were isolated from two different rice varieties, a salt tolerant variety Pokkali and an intermediate variety Nipponbare, by a homology-based screen using the *TaHKT2;1* cDNA as probe in a high stringency condition (Horie et al. 2001). The *OsHKT2;1* (previously named *OsHKT1*) cDNA was isolated from both varieties with no amino acid difference in the encoded proteins, while the *OsHKT2;2* (previously named Po-*OsHKT2*) cDNA was found only in the

rice cultivar, Pokkali (Horie et al. 2001). OsHKT2;2 was found under low Na^+ concentration to mediate TaHKT2;1-like Na^+/K^+ co-transport, whereas OsHKT2;1 was found to mediate Na^+ selective transport in yeast mutants and *Xenopus* oocytes, despite the fact that OsHKT2;1 and OsHKT2;2 are highly homologous sharing more than 91% identity at the amino acid level (Horie et al. 2001). Interestingly, *OsHKT2;1* mRNA showed a remarkable accumulation in roots of Nipponbare plants in response to the deprivation of K^+ for which OsHKT2;1 shows extremely poor permeability (Horie et al. 2001). Moreover, the K^+ deprivation-induced accumulation of *OsHKT2;1* mRNA was significantly suppressed upon the addition of 30 mM NaCl (Horie et al. 2001). Accumulations of *OsHKT2;1* and *OsHKT2;2* mRNA in Pokkali were also shown to be regulated in a similar manner in response to various Na^+/K^+ conditions as in the case with *OsHKT2;1* in Nipponbare (Horie et al. 2001). Reductions of *OsHKT2;1* transcripts in response to 150 mM NaCl in *indica* cultivars were also reported by Golldack et al. (2002). These analyses suggested that Na^+ transport through *OsHKT2;1* and *OsHKT2;2* in rice are closely controlled by K^+ status in K^+-starved rice plants and that the expression is controlled by available levels of K^+ and Na^+ (Horie et al. 2001). It has long been known in classical plant physiological studies that acquisition and maintenance of K^+ during salt stress is important for plant salt tolerance (Flowers and Läuchli, 1983 and references there in). Characterization of the salt overly sensitive 1 (*sos1*) mutant of *A. thaliana* further supported that high-affinity K^+ uptake under salinity stress is a crucial factor to maintain resistance (Wu et al. 1996). Garciabeblás et al. (2003) found in databases of *japonica* Nipponbare and *indica* ssp. that the *OsHKT2;2* gene in these varieties is a pseudogene and includes insertion of a 3.1 kb fragment of "junk" DNA sequence. Kader et al. (2006) have reported that the accumulation of *OsHKT2;2* mRNA is increased in response to salt stress in Pokkali but not in the salt sensitive variety BRRI Dhan29. It is still an open question whether the function of OsHKT2;2 could be related to the salt tolerant phenotype of Pokkali plants.

A major role for defined HKT transporters in protecting leaves from sodium damage

TaHKT2;1 homologs have been characterized or identified in many other plant species such as Arabidopsis, eucalyptus, ice plant, barley, poplar, reed and Suaeda (Fairbairn et al. 2000; Uozumi et al. 2000; Su et al. 2003; Haro et al. 2005; Gierth and Mäser, 2007). AtHKT1;1 in *A. thaliana* has been shown to mediate Na^+ selective transport in yeast and *Xenopus* oocytes (Uozumi et al. 2000). AtHKT1;1 is expressed in the vasculature of *A. thaliana* (Mäser et al. 2002a). AtHKT1;1-mediated Na^+ selective transport was later found to play an essential role in the protective mechanism against salinity stress in *A. thaliana* plants (Mäser et al. 2002a; Berthomieu et al. 2003; Gong et al. 2004; Sunarpi et al. 2005; Horie et al. 2006). Detailed protein localization analyses of McHKT1;1 and AtHKT1;1 showed that they are expressed in the plasma membrane of xylem parenchyma cells (Su et al. 2003; Sunarpi et al. 2005). Promoter-reporter gene analyses also showed the close rice homologue OsHKT1;5 (SKC1) and AtHKT1;1 expression in xylem parenchyma cells (Ren et al. 2005; Sunarpi et al. 2005). Furthermore, data showed that the *A. thaliana* AtHKT1;1 transporter and OsHKT1;5 function in removing Na^+ from the xylem sap of plants, thus counteracting Na^+ over-accumulation in leaves (Ren et al. 2005; Sunarpi et al. 2005; Horie et al. 2006). The model for the function of these HKT isoforms resulting from these studies is that under Na^+ stress conditions Na^+ levels increase in the xylem sap (Ren et al. 2005; Sunarpi et al. 2005; Horie et al. 2006). The AtHKT1;1 and OsHKT1;5 transporters can then remove Na^+ from the xylem sap (Ren et al. 2005; Sunarpi et al. 2005; Horie et al. 2006), thus prohibiting the over-accumulation of Na^+ in leaves (Mäser et al. 2002a; Berthomieu et al. 2003; Sunarpi et al. 2005), indicating that in wildtype plants the Na^+ removed from the xylem sap can be re-circulated by the phloem. Thus these studies have described a mechanism by which expression of defined HKT transporters in rice and Arabidopsis in the xylem parenchyma protects leaves from Na^+ damage. As the photosynthetic machinery in leaves is particularly sensitive to and damaged by elevated Na^+ levels (e.g. Horie et al. 2006), these HKT transporters provide an important mechanism mediating Na^+ tolerance.

Structure of the K^+ channel-like selectivity filter in the HKT-type transporters/channels determines the K^+/Na^+ selectivity

Interestingly, HKT-type transporters/channels can roughly be divided into two different groups based on the K^+ selectivity in heterologous expression systems. Ion selectivity analyses of *TaHKT2;1* and *AtHKT1;1*-chimera constructs in yeast and *Xenopus* oocytes have led to the identification of an important region in the N-terminus of TaHKT2;1, that functions in modulating K^+ selectivity (Mäser et al. 2002b). The region was found to contain a putative pore-forming region called "P-loop" that is distantly homologous to that of the bacterial K^+ channel, KcsA (Durell and Guy, 1999; Durell et al. 1999; Mäser et al. 2002b). The alignment of the P-loop regions of HKT, bacterial transporters, Trk and KtrB, and shaker K^+ channel of *Drosophila* showed that the critical amino acid that determined K^+ permeability in the above chimeras corresponded to a glycine residue in the P-

loop, which corresponds to the first glycine of the GYG motif that is highly conserved among K^+ channels for K^+ selectivity (Mäser et al. 2002b). Interestingly, the glycine residue was conserved in K^+ permeable HKTs such as TaHKT2;1 and OsHKT2;2. However, this amino acid position was serine instead of glycine in Na^+ selective HKTs such as AtHKT1;1 and OsHKT2;1 (Horie et al. 2001; Mäser et al. 2002b). HKT and its homologous transporters, Trk and KtrB, have been found to include four P-loop regions in their amino acid sequences (Durell and Guy, 1999; Durell et al. 1999). Glycine residues in the other three P-loops of HKT/Trk/KtrB transporters/channels were completely conserved, independently of the ion selectivity. These data showed that K^+ permeable HKT transporters analyzed have a G-G-G-G motif in the four P-loops, whereas the HKTs showing more Na^+ selective Na^+ transport have a S-G-G-G motif in the P-loops. Note that Na^+ "selective" in biology is always a relative term, as other related cations usually show some degree of relative permeability. Site direct mutagenesis was performed on *TaHKT2;1*, *AtHKT1;1*, *OsHKT2;1* and *OsHKT2;2* cDNAs, which converted the glycine to serine (TaHKT2;1 and OsHKT2;2) or the serine to glycine (AtHKT1;1 and OsHKT2;1) in the first P-loop region (Mäser et al. 2002b). The substitutions of the glycine to serine in TaHKT2;1 and OsHKT2;2 did not remove Na^+ transport activity but impaired their K^+ permeability in heterologous expression systems with yeast (*Saccharomyces cerevisiae*) (Mäser et al. 2002b) (Fig. 1). On the other hand, the substitutions of the serine to glycine conferred K^+ permeability to AtHKT1;1 and OsHKT2;1 leaving their Na^+ transport activity in heterologous expression systems (Mäser et al. 2002b) (Fig. 1). Consistent results were found in the analyses of Na^+-dependent K^+ transporter KtrAB complex by mutating all four glycine residues in the four p-loops of this bacterial K^+ transporter (Tholema et al. 2005). This study showed that the presence of the glycine residue in each of the four p-loops is essential for K^+-Na^+ co-transport of the wildtype KtrAB transporter, whereas mutations of these glycines led to isoforms with reduced K^+ transport and enhanced Na^+ transport (Tholema et al. 2005). Taken together, these studies demonstrated that the conserved glycine residue in the first P-loop region of the HKT/Trk/KtrB family is essential for K^+ permeability, and that the HKT/Trk/KtrB family retains similar structures of the selectivity filter, which seem to be related to those of K^+ channels (Mäser et al. 2002b).

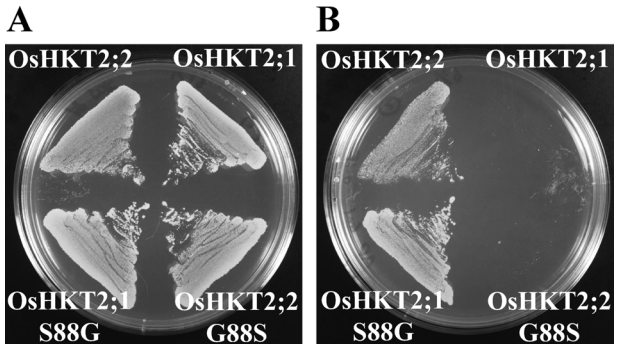

Figure 1. Conserved glycine residue in the first P-loop region is essential for K^+ permeability of HKT proteins (Mäser et al. 2002b; Copyright 2002 National Academy of Sciences, U.S.A.). Expression of OsHKT2;1 does not rescue the growth defect of high-affinity K^+ uptake deficient mutant (*trk1*, *trk2*) of *Saccharomyces cerevisiae*, CY162 strain, under a low K^+ condition, but in contrast, OsHKT2;2 does. Mutations of ser-88 to glycine in OsHKT2;1 (OsHKT2;1 S88G) and gly-88 to serine in OsHKT2;2 (OsHKT2;2 G88S) converted their K^+ permeability in the mutant cells. (A) CY162 cells expressing either OsHKT2;1, OsHKT2;2, OsHKT2;1 S88G, or OsHKT2;2 G88S on the arginine phosphate (AP) medium containing 50 mM KCl. (B) Same CY162 cells as in A, growing on the AP medium containing 0.1 mM KCl.

K^+/Na^+ selectivity of OsHKT proteins

We found that the expression of the G-G-G-G type mutant of OsHKT2;1 (OsHKT2;1- S88G) rendered salt sensitive yeast cells (G19 strain of *S. cerevisiae*) far less sensitive to Na^+ under various NaCl stress conditions presumably due to improved K^+ transport and reduced toxic Na^+ influx (data not shown). K^+/Na^+ selectivity of three kinds of OsHKT proteins, OsHKT2;1, OsHKT2;2 and OsHKT2;1-S88G, was comparatively evaluated with using a yeast assay system. We expressed each *OsHKT* cDNA under the yeast *GAL1* promoter, and introduced the chimera constructs into yeast W303 strain that retains high-affinity K^+ uptake system encoded by the *TRK1* and *TRK2* genes, since we also intended to test whether the OsHKT2;1-S88G protein could ameliorate salt tolerance of wildtype cells as a model case toward molecular breeding of salt tolerance making use of OsHKT proteins. All transgenic yeast cells grew normally in the low potassium (0.1 mM K^+) and low sodium (25 μM) condition. 100 mM of Na^+ decreased the growth of yeast cells expressing OsHKT2;1 or OsHKT2;2 by approximately 57%, however, this growth inhibition was about 12% in the case of yeast cells producing OsHKT2;1-S88G (data not shown). These results suggest that Na^+ transport activity of OsHKT2;1-S88G is weaker than OsHKT2;1 and OsHKT2;2 in the presence of excessive Na^+. To confirm this, intracellular K^+ and Na^+ contents in yeast were measured with the inductively coupled plasma optical emission spectrometry (ICP-OES). Na^+/K^+ ratios in cells expressing OsHKT2;1 and OsHKT2;2 were much higher than ratios in cells expressing OsHKT2;1-S88G in the presence of 100 or 300 mM of Na^+ (Fig. 2). However, these results are not sufficient to discuss about enhancement of salt tolerance by OsHKT2;1-S88G in yeast cells. Finding mutations which further reduce Na^+ transport of the OsHKT2;1-

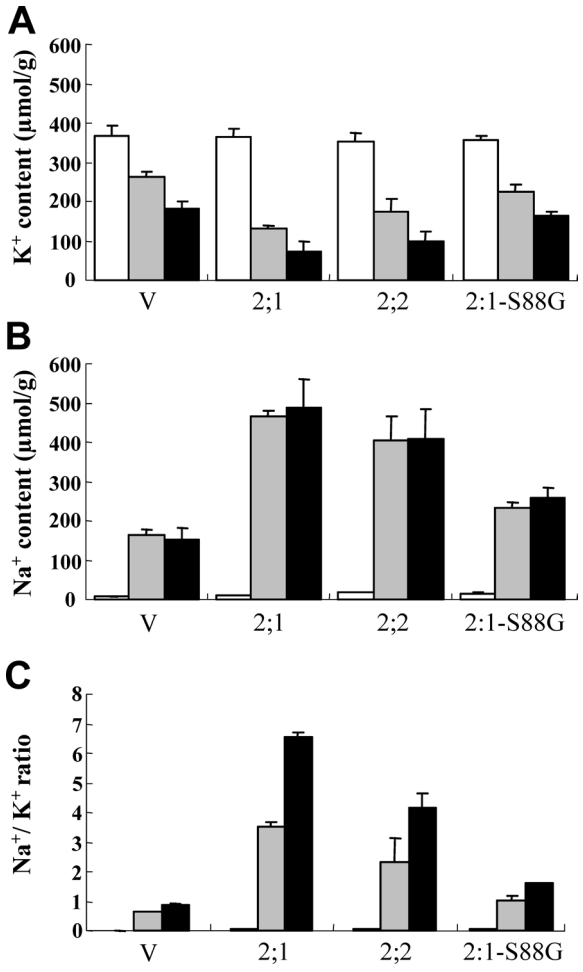

Figure 2. Intracellular K^+ and Na^+ accumulation of yeast cells expressing *OsHKT* genes. Strain W303 of *S. cerevisiae* (*MATa, trp1, leu2, ade2, ura3, his3, lys2, can1*) was used as host cells. Yeast was grown in AP medium supplemented with no NaCl (open bar), 100 mM NaCl (grey bar) or 300 mM NaCl (filled bar). K^+ content (A) and Na^+ content (B) of yeast expressing each *OsHKT* gene (2;1, 2;2 and 2;1-S88G) or vector (V) was measured with ICP-OES (IRIS Intrepid II, Thermo Fisher Scientific, USA). Na^+/K^+ ratio is calculated based on data of K^+ and Na^+ contents (C). Error bars represent ± SD (n = 3).

S88G protein leaving its K^+ transport property as it is in high Na^+ concentrations could provide an ideal tool for future molecular breeding of salt tolerant plants.

OsHKT2;1 mediates "nutritional Na^+ absorption and distribution" in K^+-starved rice plants: role of Na^+ as a substitute nutrient for K^+

OsHKT2;1 was well-characterized as a Na^+ selective transporter/channel using heterologous expression systems (Horie et al. 2001; Garciabeblás et al. 2003). However, the physiological role of OsHKT2;1 *in planta* remained to be elucidated until recently. Horie et al. (2007) found *Tos17*-insertion mutants, which are disrupted in the *OsHKT2;1* gene of Nipponbare plants. *Tos17* is an endogenous retrotransposon in rice plants, which undergoes local transposition events only during tissue culture, and thus is an ideal mutagen (Hirochika, 1997, 2001; Miyao et al. 2003). Three independent *oshkt2;1* knock out mutant alleles were isolated and grown under various Na^+ conditions to search for physiological and stress phenotypes. Interestingly, all *oshkt2;1* mutant plants were found to show remarkably meager growth under a K^+-starved and 0.5 mM Na^+-containing condition compared to wildtype (WT) plants (Horie et al. 2007) (Fig. 3). *oshkt2;1* mutants were also shown to accumulate much less Na^+ in shoots, roots and the xylem compared to WT plants (Horie et al. 2007). Furthermore, tracer influx experiments using $^{22}NaCl$ and *oshkt2;1* mutant and WT plants indicated that: (i) OsHKT2;1 directly mediates Na^+ influx into roots of K^+-starved rice plants; (ii) OsHKT2;1-dependent Na^+ influx is detected only in K^+-starved roots (not with K^+-supplied roots); (iii) Approximately 80 to 90% of Na^+ influx into intact K^+-starved rice roots is mediated by OsHKT2;1-dependent Na^+ influx at low Na^+ concentrations (1 to 200 μM); and (iv) OsHKT2;1 robustly mediates Na^+ influx at low millimolar Na^+ concentrations, although the relative contribution of OsHKT2;1 to Na^+ influx to whole root Na^+ influx is reduced at an increase in the Na^+ concentration, presumably because of the effect of the other Na^+ permeable transporters/channels working at higher Na^+ concentrations in rice roots (Horie et al. 2007). Furthermore, whereas *oshkt2;1* knock out mutations dramatically reduced Na^+ influx into roots, Rb^+ influx was not greatly affected (Horie et al. 2007), consistent with the high Na^+ to K^+ selectivity of OsHKT2;1 measured in heterologous systems (Horie et al. 2001).

The above results demonstrated that Na^+, which is not an essential nutrient for plant growth and causes toxicity when it is excessively accumulated in plants, can substitute for some roles of K^+ as a substitute nutrient in K^+-starved rice plants (Horie et al. 2007). Moreover, the results also clearly demonstrated that the OsHKT2;1 transporter/channel is responsible for the nutritional Na^+ uptake into K^+-starved roots and distribution throughout K^+-starved rice plants (Horie et al. 2007) (Fig. 4A). Classical plant physiological studies have pointed out that Na^+ behaves as a substitute nutrient for K^+ under K^+-starvation in some plants (Mengel and Kirkby 1982; Flowers and Läuchli 1983 and references there in). The report of Horie et al. (2007) provides robust genetic evidence that Na^+ indeed substitutes for some roles of K^+, and that the gene causing this phenomenon is the *OsHKT2;1*-type gene *in planta*. It should be mentioned that not every plant species shows positive effects upon the addition of Na^+ under K^+-starved conditions, and that plants seem to be categorized into four groups, depending on the response when Na^+ is added (Flowers

Figure 3. *oshkt2;1* mutant plants show significant growth defect when a moderate amount of Na^+ exists in K^+-starved condition. An example of growth assays using *oshkt2;1-1* allele, which is one of three *oshkt2;1* mutant alleles reported in Horie et al. (2007). Ten-day old K^+-starved plants were transferred onto hydroponic solution which contains no K^+ and 0.5 mM Na^+. The picture was taken 15 days later. Note that TosWT2;1-1 represents corresponding wildtype plants, which were isolated from the same parent plants as *oshkt2;1-1*.

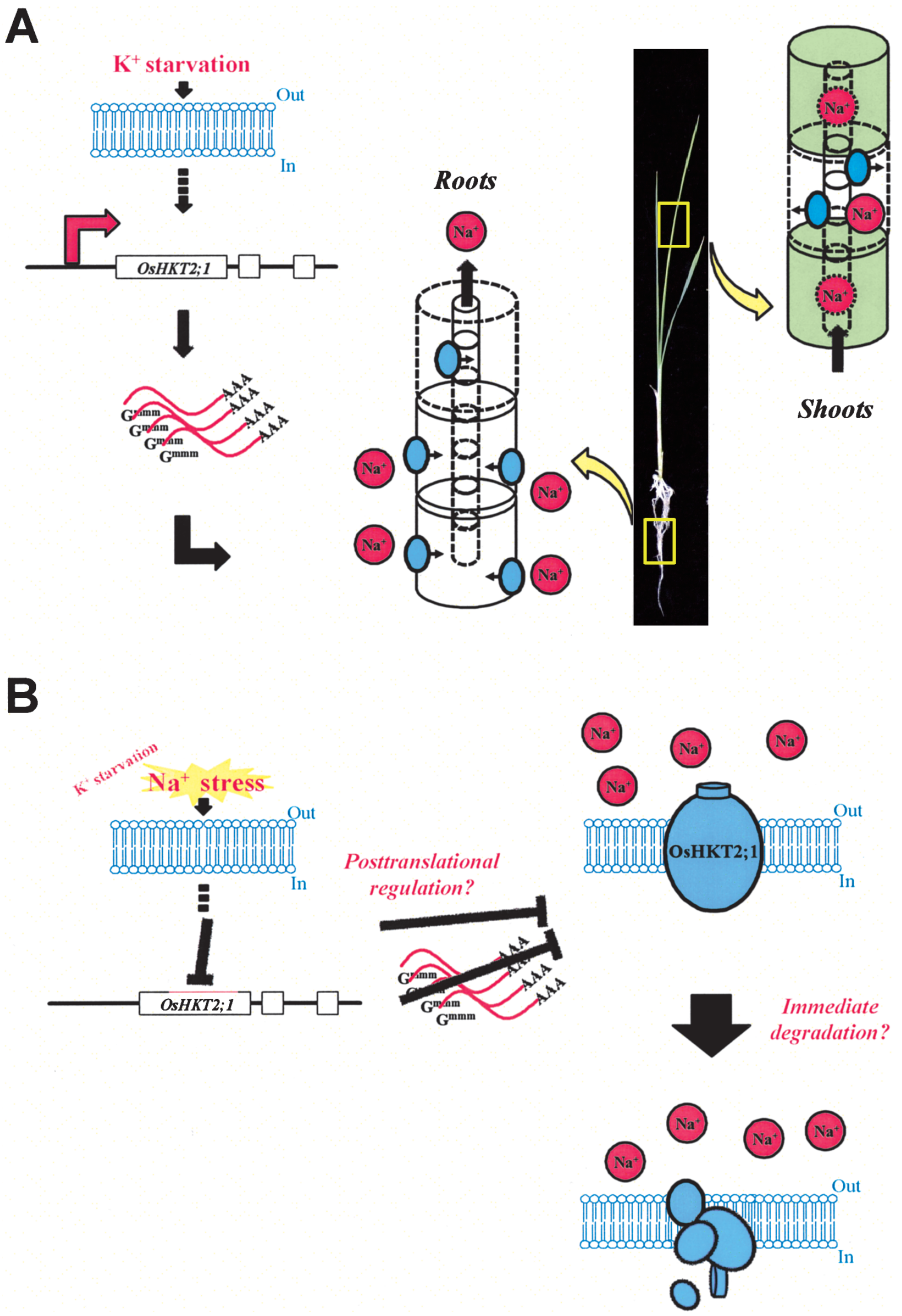

Figure 4. Models of the function of OsHKT2;1 in nutritional Na^+ uptake and rapid repression of OsHKT2;1 in the presence of a large amount of Na^+. (A) Transcription of the *OsHKT2;1* gene is activated in response to K^+ starvation. OsHKT2;1 functions in Na^+ absorption and distribution in K^+-starved rice plants for growth. (B) OsHKT2;1-dependent Na^+ influx is rapidly down-regulated in response to high concentrations of Na^+ by means of transcriptional and most likely rapid posttranslational regulation mechanisms (Horie et al. 2007).

and Läuchli, 1983).

OsHKT2;1 has been shown to mediate large Na^+ influx into cells with a Na^+ channel-like feature in heterologous expression systems (Horie et al. 2001), which could lead to the hypothesis that OsHKT2;1 mediates toxic Na^+ influx under saline conditions. However, *oshkt2;1* mutant plants did not show any difference under salt stress compared to WT plants (Horie et al. 2007). Interestingly, $^{22}NaCl$ tracer influx experiments indicated that OsHKT2;1-dependent Na^+ influx is rapidly down-regulated by the addition of 30 mM NaCl on K^+-starved roots (Horie et al. 2007). These results suggest that OsHKT2;1-dependent Na^+ influx in K^+-starved roots is tightly regulated to prevent Na^+ toxicity due to mass flow of Na^+ via OsHKT2;1 (Figure 4B). Elucidating the molecular mechanism that

posttranslationally controls the activity of OsHKT2;1 could provide important clues and hints for developing new technology toward future molecular breeding of salt tolerant plants.

Roles for other plant HKT transporters

The HKT-type transporter/channel is one of the most studied Na^+ permeable proteins in plants (Horie and Schroeder 2004). Nine *OsHKT* genes including *OsHKT2;1* have been identified on the genome of Nipponbare plants and two of them were further found to be pseudogenes, suggesting that 7 full-length *OsHKT* genes are expressed in Nipponbare plants (Garciabeblás et al. 2003). It is worth paying attention to the fact that *oshkt2;1* mutant plants showed strong phenotypes in nutritional Na^+ absorption and distribution nevertheless the other 6 *OsHKT* genes are functional. The *OsHKT2;1* gene may be a specific gene evolved to maintain alkali metal cation homeostasis of rice plants under poor soil conditions (Horie et al. 2007; Mueller-Roeber and Dreyer 2007). Among 6 other *OsHKT* genes, *OsHKT1;4* and *OsHKT1;5* (*SKC1*) are close homologs of *AtHKT1;1* which is essential for salt tolerance of *A. thaliana* plants. Furthermore, from rough mapping studies, *HKT1;4* and *HKT1;5* alleles of durum wheat were reported as putative candidates genes in the mapping regions conferring salt tolerance to wheat plants (Huang et al. 2006; Byrt et al. 2007). These wheat genes are also the closest homologs to *AtHKT1;1* and thus appear to have an orthologous function to that characterized in *A. thaliana*. We do not yet have any evidence for the physiological roles of OsHKT1;1, 1;3, 2;3 and 2;4 transporters in rice plants. Investigating their roles will be important future inquiries to figure out the complete mechanism of Na^+ homeostasis through OsHKT proteins in rice plants.

Conclusions

Salt stress is one of the most relevant abiotic stresses reducing agricultural productivity. Soil salinity is expanding in irrigated lands, which is a serious threat to agriculture. HKT-type transporters/channels have been shown to play essential roles in Na^+ homeostasis in plants, such as salinity resistance and growth under K^+-starved conditions. Elucidating roles of Na^+ permeable transporters/channels including HKT proteins in salinity resistance is an essential subject to address. Global climate change and its potential impact on food production may threaten life of mankind. The necessity to elucidate the whole picture of resistance mechanisms to abiotic and biotic stresses in plants and to develop technology for producing stress resistant plants is of great relevance for agriculture in the 21st century.

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