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GENOTYPIC CHARACTERIZATION AND TISSUE LOCALIZATION OF THE MUTANT LINES EXPRESSION OF *HKT1;3* GENE IN RICE UNDER SALT STRESS

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Abstract

In rice (*Oryza sativa* 'Nipponbare'), the genes family HKT is composed of eight members. It encodes for Na^+ transporters that play an important role in salt stress tolerance. Functional analysis, it has been determined that there is a wide diversity of these transporters in terms of their Na^+/K^+ selectivity and K^+ affinity, in addition, plays an important role in reducing Na^+ accumulation in shoots to cope with salt stress. This study focuses on one of these genes, yet to be characterized in literature: *OsHKT1;3* (also known as HKT6). Using reverse genetics, the aim of this study is to determine the role of this gene during salt stress. In order to observe the expression of this gene in different organs of the plant, GUS transcriptional fusions were made, with GUS as the reporter gene. Then, using the CRISPRCAS9 method, previously, loss of function mutant lines were generated. Physiological test were made on effect of loss of function of *OsHKT1;3* on Na^+ and K^+ accumulation in rice tissues during salt stress on different tissues (roots, sheath and leaf blade). The results obtained show that all mutants are homozygous, induce a frame shift and being present at the beginning of the gene (1st exon). *OsHKT1;3*::GUS showed strong GUS activity, expressed mainly in vascular tissues and did not show a significant difference in the expression with the NaCl- and water-treated parts. In contrast, the GUS activity of the *OsHKT1;3* promoters in NaCl-treated leaves was greater than that in water-treated leaves. The results of this study show that in wild plants, increasing the Na^+ concentration in the culture medium has the effect of increasing the Na^+ content of the tissues generally, the old leaves accumulating more Na^+. When the Na^+ concentration increases, the K^+ content decreases in roots and old leaves, but varies little in young leaves. Analysis of Na^+ storage in tissues also shows that Na^+ levels are higher in the lower parts of the leaf than in the upper parts. Analysis of Na^+ storage in tissues also shows a Na^+ in leaf blade more evenly distributed among the first leaves than in WT plants. Taken together, these results suggest that *OsHKT1;3* gene plays a role in the accumulation of Na^+ in old leaves.

Key words : *Oryza sativa*, salt stress, OsHKT1;3, CRISPR-CAS9, GUS.

Introduction

Soil salinity is one of the abiotic stresses limiting the growth of crops, which results in lower yields. This makes it one of the main environmental constraints facing modern agriculture. Moreover, often associated with drought, it leads to a reduction of arable land and threatens the global food balance. The model plant used, rice (*Oryza sativa*), is a species sensitive to salt, like most cultivated species. The cultivar used is Nipponbare.

Resistance to salt stress involves complex mechanisms including the control of the activity of many transporters, in particular transport of Na^+ and K^+. Indeed, salt tolerant species are characterized by the ability to effectively accumulate K^+, even in the presence of high Na+ concentrations, and by controlling the spatial distribution of Na^+ accumulation in the shoots. HKT Na^+ transporter with 8 members in japonica rice (Garciadeblás *et al*., 2003) play an important role in this resistance (Munns *et al*., 2012; Ren *et al*., 2005). Functional analyzes show a significant diversity of this transporter with respect to their Na^+/K^+ selectivity and Na^+ affinity (Sassi *et al*., 2012; Jabnoune *et al*., 2009). The different members of the HKT family in rice therefore probably play different roles in the physiology of the plant and the adaptation to salt stress.

Localization studies by analysis of transformed plants

with a fusion of promoter::GUS have shown that *OsHKT2; 1* and *OsHKT1; 5* are expressed in the vascular tissues. More precisely, reveal that *OsHKT2;1* is localized to the vascular tissues of leaves but also in the root cortex and endoderm (Horie, 2007), eventhough, it is mainly localized in the xylem parenchyma, in the roots and in the leaves (Ren, 2005).

The objective of this work is to clarify the role of *HKT1;3* gene under saline stress by an inverse genetic approach. This gene encode selective Na^+ transporter. In a heterologous expression system, *HKT1;3* has an affinity for Na^+ (Jabnoune *et al*., 2009). Mutant rice loss lines were isolated for this HKT gen using CRISPR-CAS9 technology, which is today widely used to induce targeted (double-stranded) changes in different genomes in animals and plants, particularly in rice because of its simplicity, efficiency and versatility (Pan *et al*., 2016; Jacobes *et al*., 2015; Shan *et al*., 2014; Miao *et al*., 2013). As a result (CRISPR associated protein-9 nuclease) has become a tool of genetic engineering to improve crops by creating differences in germplasm compared to other technologies used to modify genomic DNA such as ZFNs and TALENs (Hsu *et al*., 2012; Urnov *et al*., 2010), but this technology is more specific than the others because it targets the desired DNA site by double-strand cutting at the same time. The phenotype of these mutants lines is analyzed during a salt stress.

Materials and Methods

Vegetal material

The variety used is a japonica rice cultivar Nipponbare as a plant material. The rice is grown under hydroponic conditions in Yoshida medium. The plants used are one month old. The plants are stressed under 2 conditions: addition of 50 mM or 150 mM NaCl to the culture medium for 3 days. The control condition corresponds in Yoshida medium. For this study, second generation homozygous plants from three CRISPR lines were used: *HKT1;3: HKT1;3.2.1, HKT1;3.4.2, HKT1;3.4.4.*

DNA extraction and PCR experiment

The genomic DNA is extracted from small pieces of leaves and a fragment of the HKT1;3 gene is amplified by PCR using the "Phire Plant Direct PCR" KIT Thermo Scientific. To carry out the PCR reaction, a reaction mixture is prepared and added to the plant DNA extract. The PCR amplification program is as follows: initial denaturation for 5 min at 98 °C followed by 40 cycles of amplification comprising a denaturation step of 5 sec at 98°C, hybridization of the primers for 5 sec to 55°C and elongation for 20 sec at 72°C. A final elongation step at 72°C is carried out and followed by cooling to 4°C. The size of the amplicons obtained is then observed after migration by electrophoresis on a 1% agarose gel in a 0.5X TAE buffer. SYBR Green is used as a DNA intercalator. This is revealed using ultraviolet light. The amplified DNAs are then purified with a Thermo Scientific "GeneJET PCR Purification Kit" kit and sent to sequence.

GUS Staining and Activity Assay

Histochemical activity of GUS in transgenic plant materials and quantitative analysis of GUS activity it includes 4 steps using different solutions and was detected according to the method of Jefferson *et al*. (1987).

Tissue analysis

With regard to tissue analysis, stressed plants are separated into 3 types of samples: root, foliar sheath and leaf blade. The Na^+ and K^+ contents of the samples being too high in the tubes of 5 ml HCl 0.1 N. Then, the mean of the results for the plants of the different genotypes is calculated. The assays of the Na^+ and K^+ elements are carried out by atomic absorption spectrometry.

Results

Verification of the loss of function character of mutant lines in genes OsHKT1;3

Sequencing of the amplified OsHKT1;3 gene fragments (fig. 1) confirms the mutations observed in previous generations (fig. 2): (HKT1;3.2.1: - CTTA, HKT1;3.4.2: + C, HKT1;3.4.4: + 8 bp). All mutations induce a frameshift and being present at the beginning of the gene (1st exon), they cause a loss of function of the gene. All mutants are homozygous. The control line shows no mutations in this gene.

Tissue localization of expression

The results of the histochemical GUS staining in vitro under control conditions showed elevated GUS localization in leaves, mainly in vascular tissues, including phloem and xylem (figs. 3a and 3b) and also in vascular tissues of sheaths (figs. 3c and 3d), GUS activity was detected mainly strong in vascular tissues of roots (figs. 3e, 3f and 3g) and was seemed clear in the vein of flower (vascular tissues) (fig. 3h).

Expression analysis of OsHKT1;3 promoter::GUS in transgenic rice plants in response to NaCl (50 mM) treatment showed elevated GUS localization in leaves, GUS staining was detected in phloem and epidermis (figs. 4a and 4b) and in the sheaths, a strong GUS coloration was seemed clear in the xylem, phloem and the parenchyma cells (figs 4c and 4d). GUS activity in the

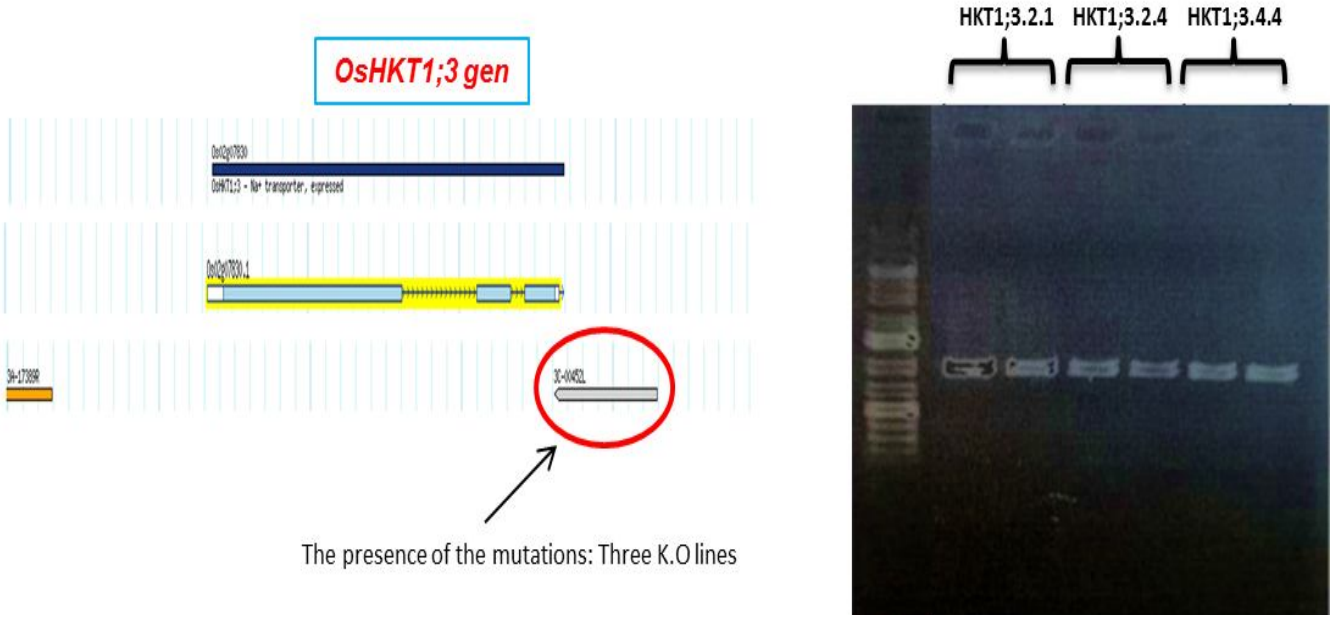

Fig. 1 : Verification of HKT1; 3 mutants by PCR amplifications.

CrHKT1;3.2.1

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        300       310       320       330       340       350
sgARN2 TTCTAATAACAAACTTAGGCGGCTCCCTACTTATTTACCTCTAC----ACCTGGTACCAA
       :::::::::::::::::::::::::::::::::::::::::::    ::::::::::::
OsHKT6 TTCTAATAACAAACTTAGGCGGCTCCCTACTTATTTACCTCTACCTTAACCTGGTACCAA
      330       340       350       360       370       380
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CrHKT1;3.4.2

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          500       490       480       470       460       450
sgARN2 TATTTACCTCTACCTTAAACCTGGTACCAAGTGCACATAAAATTCTAAAGAGAAAAGGCA
       ::::::::::::::::: :: ::::::::::::::::::::::::::::::::::::::::
OsHKT6 TATTTACCTCTACCTTAA-CCTGGTACCAAGTGCACATAAAATTCTAAAGAGAAAAGGCA
     360       370       380       390       400       410
```

CrHKT1;3.4.4

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          130       120       110       100        90
sgRNA4 CTTGATCATTCTGCAAACCGTATTGTTCCTCTCTTTGGAGTGGAGCT--------TTGGA
       ::::::::::::::::::::::::::::::::::::::::::::::::        :::::
OsHKT6 CTTGATCATTCTGCAAACCGTATTGTTCCTCTCTTTGGAGTGGAGCTCGGTAGCTTTGGA
     720       730       740       750       760       770
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WT CrHKT1;3

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          140       150       160       170       180       190
SgARN4 AGCGCTGTGGAAATAAAATGGCGTTATTTCTGTTTGCTTGTAAGCAACCAAAAAAGAAAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
OsHKT6 AGCGCTGTGGAAATAAAATGGCGTTATTTCTGTTTGCTTGTAAGCAACCAAAAAAGAAAA
     160       170       180       190       200       210
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Fig. 2 : Alignment of mutant sequences to observe mutations.

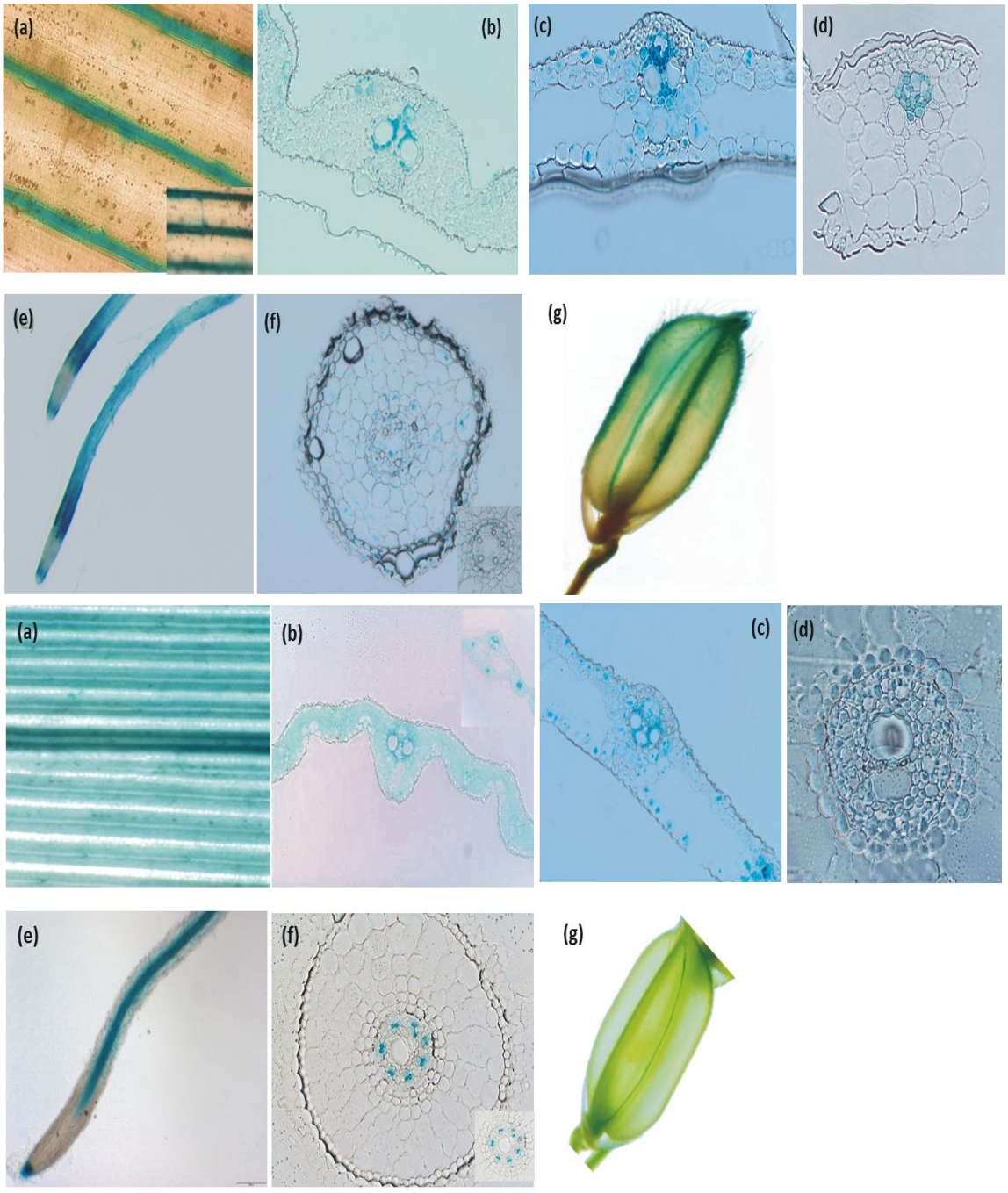

Fig. 3 : OsHKT1;3 gene expression localization in transgenic rice plants under the control conditions, in leaves (a), (b). Sheaths (c), (d). Roots (e), (f). Flower (g).

roots was shown that GUS staining very clear, strong and localized in vascular tissues (figs. 4e, 3f and 4g) and was seemed strong also in the vein of flower (vascular tissues) (fig. 4h).

Effect of loss of function of OsHKT1;3 on Na^+ and K^+ accumulation in rice tissues during salt stress

For the effect of increased Na^+ concentration in the culture medium on Na^+ and K^+ levels of tissues in control plants of wild genotype, I observed a strong increase in Na^+ content of the tissues when Na^+ in the culture medium increased from 0.3 mM (control) to 50 mM then 150 mM in wild plants (fig. 5). This increase is very high between stresses 50 mM and 150 mM NaCl. With increasing salt stress, the K^+ content decreases in the root (fig. 6). In all three conditions, except for the leaf blades of F2 whose value is certainly artifactual, there is a decrease in the K^+ content of the root to F2 or F3 (the F1 is the oldest) and then an increase in the youngest

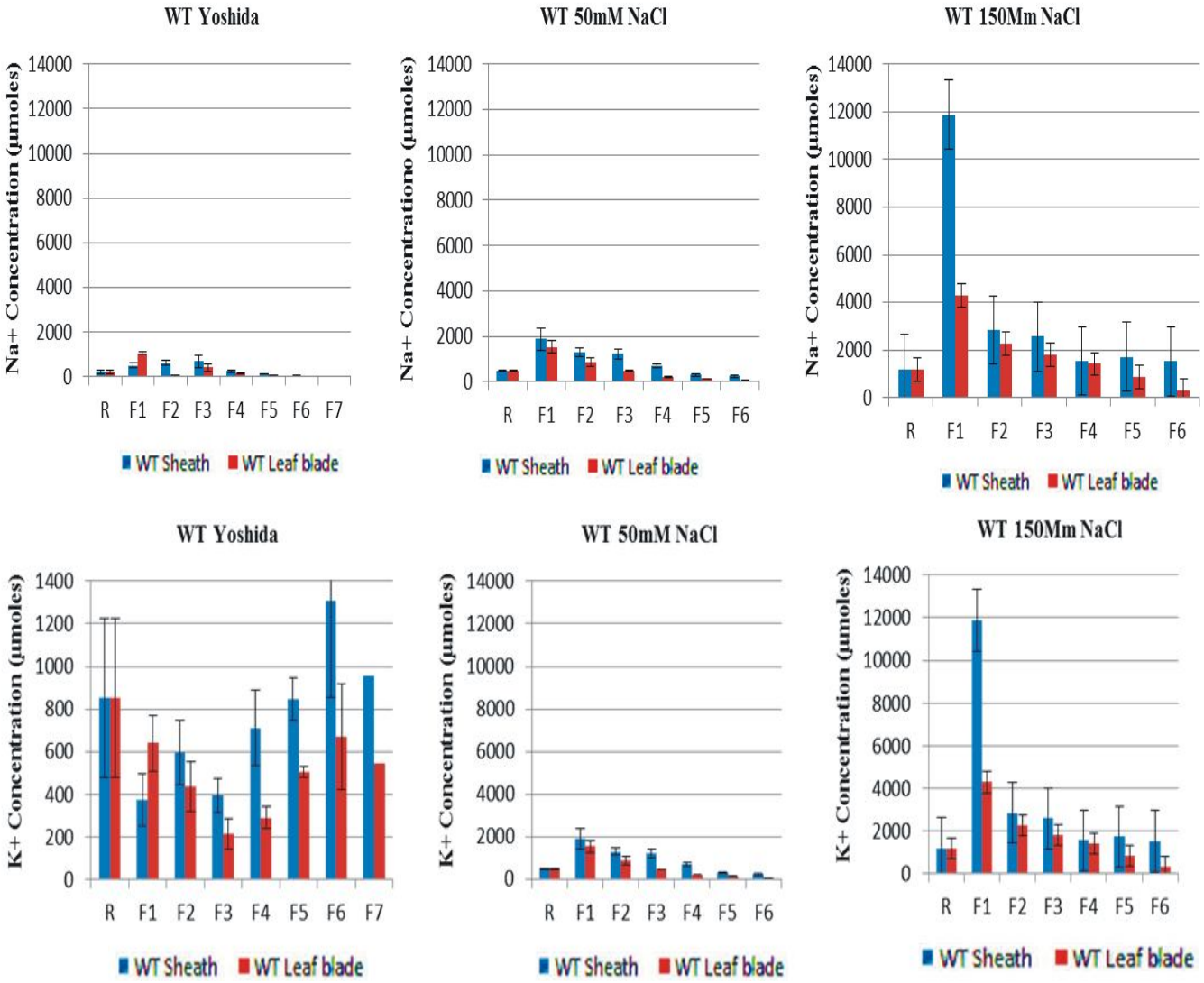

Fig. 5 : Evolution of the Na^+ content in the tissues (root, sheath or leaf blade of the different leaves) as a function of the applied stress.

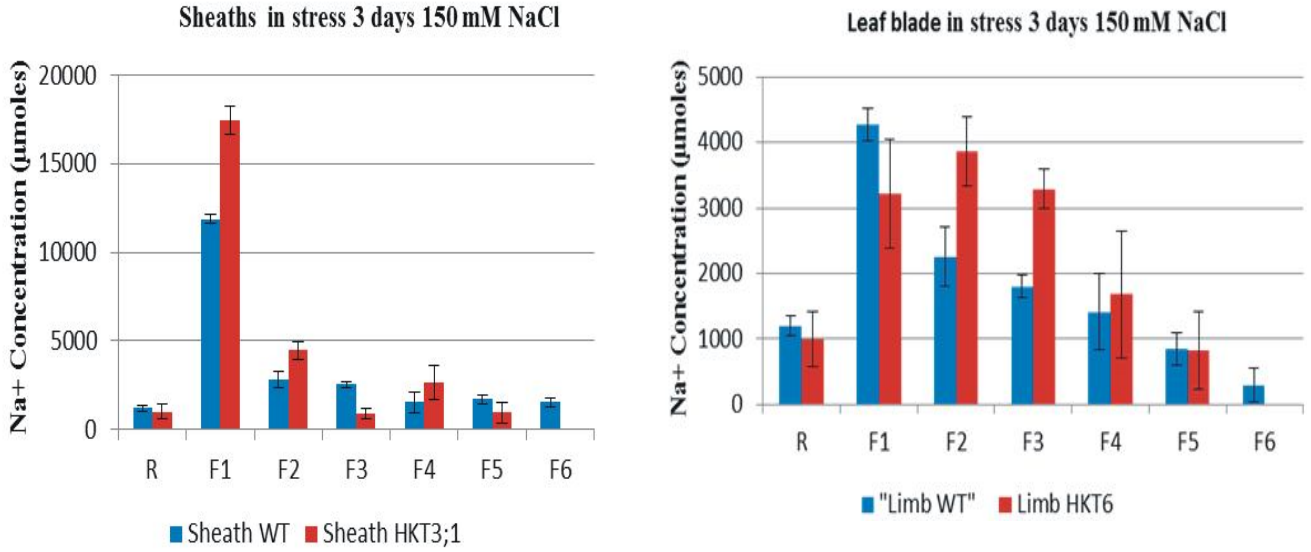

Fig. 6 : Evolution of the K^+ content in the tissues (root, sheath or leaf blade of the different leaves) as a function of the applied stress.

leaves. In addition, the K^+ content is greater in the sheaths than in the leaf blades.

As for the effect of loss of function of OsHKT1;3 on Na^+ contents of tissues at 150 mM NaCl salt stress, in stressed WT plants 3 days at 150 mM NaCl, Na^+ is accumulated mainly in the old leaves (F1 & F2), especially in the sheaths. We observed an increase in Na^+ content in the sheaths of leaves F1 and F2 in mutants. The HKT1;3 mutants show a more generalized Na+ accumulation profile in the leaf blades, with high concentrations in the first 3 leaves whereas the accumulation of Na^+ mainly concerns F1 in WT plants.

Discussion

In wild-type plants, increasing the Na^+ concentration in the culture medium has the effect of increasing the Na^+ content of the tissues generally, the old leaves accumulating more Na^+. When the Na^+ concentration increases, the K^+ content decreases in roots and old leaves, but varies little in young leaves. These results are interesting because the higher the K^+/Na^+ ratio, the better the salt tolerance of the plant. Thus, wild-type plants protect their young leaves during salt stress by accumulating more K^+ in young leaves and more Na^+ in the old leaves. Analysis of Na^+ storage in tissues also shows that Na^+ levels are higher in the lower parts of the leaf than in the upper parts, and are even more important in roots than in leaves in the case of young leaves. Analysis of Na^+ storage in tissues also shows a Na^+ in leaf blade more evenly distributed among the first leaves than in WT plants. This suggests that OsHKT1;3 plays a role in the accumulation of Na^+ in old leaves.

Conclusion

Among the objectives of this study were the genotyping of the plants used to check for the presence (or absence in the control plants) of the mutations in the targeted gene, analyzes of the localization of expression by histochemical tests of the activity GUS, phenotypic analyzes. Predominant expression in vascular tissues was observed. Phenotypic analysis revealed changes in Na^+ accumulation profiles in mutants, lack of distribution of Na^+ in leaf blades between old and young leaves. OsHKT1;4 (HKT7) and OsHKT1;5 (HKT8) had previously been identified as playing a role in the desalination of young leaves (Suzuki *et al*, 2016; Ren *et al*, 2005). The results of this study identify another HKT in rice involved in this desalting. They also show differences in mechanisms contributing to the desalting of young leaves, OsHKT1;4 occurring only at the vegetative stage, and OsHKT2;1 (HKT1), essentially at the roots. The good understanding of OsHKT1;3 gene is important, as it is one of the last two subfamily 1 genes in Nipponbare not yet well known (with OsHKT1;1). Thereafter, the mechanisms that control the accumulation of Na^+ in rice can be better understood. The characterization of HKTs entering the K^+ permeability can be done and then directly linked to those controlling the Na^+ accumulation, because HKT2;1 is currently characterized in the plant.

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