Phenotypic Characterization and Determination of Gene expression of Genetically Modified Rice Strains Using CRISPR-CAS9 Technology With Sodium Chloride Effect

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Abstract

This study was conducted with the target of determine the role of OsHKT4 and OsHKT6 genes in rice plants under salt stress and observe its gene expression by GUS technology, as well as studying the Na\textsuperscript{+} and K\textsuperscript{+} accumulation in different tissues. The results obtained show that OsHKT4::GUS appeared strong GUS activity, expressed mainly in vascular tissues. In contrast, the GUS activity of the OsHKT6 promoters in NaCl-treated leaves was greater than that in water-treated leaves. Also in wild type plants, increasing the Na\textsuperscript{+} concentration has the effect of increasing the Na\textsuperscript{+} content of the tissues generally, the old leaves accumulating more Na\textsuperscript{+} which reduced the K\textsuperscript{+} content in roots and old leaves (Na\textsuperscript{+} levels are higher in the leaf lower parts). These results suggest that OsHKT4 and OsHKT6 genes plays a role in the accumulation of Na\textsuperscript{+} in old leaves, by adopting the mechanical exclusion of toxic ions in the old leaves of the plant.

Keywords: CRISPR-Cas9, GUS, HKT, Rice, Salinity

1. Introduction

Soil salinity is one of the main abiotic constraints in agricultural production. This problem has exacerbated over the last 25 years due to the increase in irrigation needs in arid and semi-arid regions. Irrigation is often practiced with poor quality water, which leads to progressive soil salinization, which causes significant losses in crop production every year [1, 2]. In Iraq, about 60\% of potential rice-growing areas are affected by salinity [3].

HKT transporter family comprises Na\textsuperscript{+}-selective transporters and Na\textsuperscript{+}-K\textsuperscript{+}-co-transporters that maintain K\textsuperscript{+}/Na\textsuperscript{+} homeostasis and they have an important role in increasing salinity tolerance. Functional analyzes show an important diversity of these transporters with regard to their selectivity Na\textsuperscript{+}/ K\textsuperscript{+} and their affinity for Na\textsuperscript{+} [4,5,6]. The different members of the HKT family in rice thus play different roles in plant physiology and adaptation to salt stress. OsHKT4 and OsHKT6 are two of the four HKT transporters genes of sodium-selective rice. It has been identified as playing an important role in desalting leaves, and thus the tolerances to the salt stress [7,8]. Different approaches have been adopted to understand the mechanism of salinity tolerance in rice plants, among these methods (CRISPR-Cas9) recently dominated system, it must much more precise and efficient, in addition more rapid and simple compared with the other techniques of genetic modification, and is today used to induce targeted modifications in different genomes, including plant [9,10]. GUS reporter system (5-Bromo-4, Chloro-3, Indol-β-glucuronidae acid) is a histochemical technique, and their aim is analysis the activity of a promoter in a expression of gene under the GUS promoter by visualization of its activity in different tissues [11]. The data of expression available for members of the HKT family in rice revealed relatively broad patterns of expression but however it focused on two types of tissues: the peripheral tissues of the root and the vascular tissues of the root and leaves [12,7]. The target of this study is phenotypic characterization and determination of gene expression of genetically modified rice strains using CRISPR-CAS9 technology under sodium chloride effect.
2. Materials and Methods

The variety of rice used is Japonica and the cultivar Nipponbare. Rice is grown under hydroponic conditions in a Yoshida environment. The plants used are one month old and undergo a 3-day salt stress, in order to analyse the tissues. Plants are stressed under 2 conditions: 50 mM NaCl and 150 mM NaCl. The control condition corresponds to the Yoshida medium. For this study, third generation homozygous plants from 8 lines are used:

- 2 wild lines which serve as control: WT HKT 4.1.5.2 and WT HKT 4.4.5.1
- 6 mutant lines were created with CRISPR / CAS9 technology
  - 1 HKT mutant line 4.1.3.1.2
  - 2 mutant lines HKT 4.4.14.4.1 and HKT 4.4.6.1.3
  - 1 HKT mutant line 6.2.1.1.1
  - 2 mutant lines HKT 6.4.4.1.3 and HKT 6.4.2.3.4

Each mutant line has the following mutations:

- HKT 4.1.3.1.2 : + A
- HKT 4.4.6.1.3 : + G
- HKT 4.4.14.4.1 : + T
- HKT 6.2.1.1.1 : - CTTA
- HKT 6.4.2.3.4 : + C
- HKT 6.4.4.1.3 : + A

2.1 DNA extraction of HKT4 and HKT6 genes of Pokkali cultivar

This method is carried out using the dilution buffer of the Phire® Plant Direct PCR Kit according to the protocol recommended for rapidly preparing plant DNA samples. The plant leaf sample (usually less than 1 mg) is placed in 20μl of dilution buffer. 0.5 μl of sample was used as a template for a PCR reaction with 20 μl using Phire® Hot Start II DNA polymerase (Thermo Fisher Scientific) or GoTaq G2 Green Master Mix (Promega). Our amplified DNAs are then purified with a Thermo Scientific GeneJET PCR Purification Kit (REF) and sent to sequencing.

2.2 GUS technique

2.3 Cloning a digested product

The histochemical analysis of GUS technique in transgenic plant and analysis of GUS activity it includes four steps using different chemical compounds and detected by the method of [13]. "GUS staining" is made on promoters of OsHKT4:GUS and OsHKT6:GUS lines. It consists of four steps using different solutions. The histochemical coloration was carried out according to a protocol of [14]. The observation of tissues was done under a microscope BH2 (Olympus) in white light.

2.4 Phenotypic analysis (Determination of Na⁺ and K⁺ contents)

At the end of the treatment with salt stress or standard hydroponic, the plants are classified and distributed into 3 types of samples: root, leaf sheath and leaf blade, which are excised, rinsed in deionized water, then briefly dried between two layers of paper (type paper paper) and placed in aluminium foil. The samples are dried at 60 °C for three days to allow desiccation, then the samples are then weighed using a precision balance. The Na⁺ and K⁺ ions were extracted from dried tissues by dissolving it with 5 ml of 0.1 N HCl for 24 hours. Solubilized samples were diluted with 0.1 N HCl, since the measured contents must be of the order of 1 mg/l for each samples (roots, sheaths and leaf blades) to be assayed. Finally, the dosages of Na⁺ and K⁺ elements of plant tissues were performed by atomic absorption spectrophotometry (SpectrAA 220 FS, Varian).

2.5 Tissue analysis

Stressed plants are separated into 3 types of samples: root, leaf sheath and leaf blade. It was placed in aluminum and then in an oven at 37°C for 48 h in order to dry it, samples were then weighed and placed in tubes containing 5 ml of 0.1 N HCl for it weighed and to allow ions to be extracted. Finally, the determinations of the Na⁺ and K⁺ ions are carried out by flame atomic absorption spectrometry.
3. Results and Discussion

3.1 Verification of the loss of function of the strains

Our mutants present a mutation within our genes of interest HKT4 and HKT6. This creates a phase shift and therefore a loss of function of our genes because even if there are many transcripts, these do not no longer code the right protein (reformulate). Thanks to a PCR experiment on genomic DNA, it is possible to observe the presence of the HKT 4 with a size of 2000 nucleotides and HKT 6 genes with a size of 1750 nucleotides in the different mutants (Figure 1). All of our lines have our genes of interest. Once the sequences have been received and the alignment thereof, it is possible to observe the mutations obtained if they are similar to the previous generations and the mutants are homozygous (Figure 2). We can therefore observe that the mutants are indeed homozygous and the mutations obtained in the previous generations are found here.

3.2 Tissue localization of the expression of OsHKT4 and OsHKT6

Histochemical tests of GUS activity show for HKT4 under saline stress 50 mM: in the leaf blades, a very clear coloration in the vessels, more strongly in the xylem (Figure 3,A). At the root level, GUS activity is observed in both the xylem and the phloem (Figure 3,B). For HKT6 under saline stress of 50 mM, we can observe: in the sheaths, a strong GUS coloration at the level of the xylem, phloem and parenchyma (Figure 3,C). In the leaf blade, the coloration is observable in the phloem and the epidermis (Figure 3,D).

Figure 1. PCR gel of the HKT4 (a) and HKT6 (b) rice mutants to see the good presence of the genes.

Figure 2. Alignment of the sequences of the different mutants to observe the mutations.
3.3 Effect of loss of function of OsHKT4 and OsHKT6 on the accumulation of Na+ and K+ in rice tissue under salt stress

3.3.1 Effect of increasing the concentration of Na+ in the culture medium on the tissues of Na+ and K+ contents in wild-type control

we detected a high increase in the Na+ content of the tissues when Na+ in the culture medium increases from 0.3 mM (control) to 50 mM then 150 mM (Figure 4). This increase is very strong between the 50 mM and 150 mM NaCl stresses. With the increase in salt stress, the K+ contents decrease in the root (Figure 4, Left side). In the three conditions, except for the limb of F2, the value of which is certainly artefactual, there is a decrease in the K+ content of the root at F2 or F3 (F1 being the oldest) then an increase in the younger leaves. In addition, the K+ content is greater in the sheaths than in the leaf blade.

3.3.2 Effects of loss of function of OsHKT4 and OsHKT6 on the Na+ contents of the tissues under saline stress at 150 mM NaCl

In WT plants stressed 3 days at 150 mM NaCl, Na+ is accumulated mainly in the old leaves (F1 & F2), especially in the sheaths. We observe an increase in Na+ content in the sheaths of leaves F1 and F2 in the HKT6 mutants. On the other hand, the sheaths of the old leaves show a defect in the accumulation of Na+ in the HKT4 mutant (see Figure 4, Right side).
Conclusion

The expression of OsHKT4 has been observed in vascular tissues (mainly xylem) in the roots and in the leaves, this suggests that OsHKT4 is involved in the desalination of xylem sap when it rises from the roots to the leaves. The analysis of the storage of Na⁺ in the tissues also shows an over accumulation of Na⁺ in the leaf blade in the mutants loss of function. The increased storage of Na⁺ in the leaf blade is probably due to the fact that the sheaths no longer desalinate properly as in WT plants. A strong expression of OsHKT4 at the sheaths could explain these observations. OsHKT6 is mainly expressed in the leaves, both in the xylem and the phloem. Analysis of the storage of Na⁺ in the tissues also shows an Na⁺ content in the leaf blade more evenly distributed between the first leaves than in WT plants. This results suggests that OsHKT6 plays a role in the accumulation of Na⁺ in old leaves.
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