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THE OVEREXPRESSION OF GENE ENCODING RICE POTASSIUM CHANNEL — OsTPKa INCREASES THE SALT AND DROUGHT TOLERANCE OF PLANTS

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The main aim of this study was to investigate the role of potassium TPK channel (OsTPKa) from rice in regulation of salinity and drought stresses, potassium deficiency. In order to elevate the expression level of gene encoding two-pore potassium channel OsTPKa, the stable agrobacterium mediated transformation of plants was performed.

The elevation of OsTPKa expression level in transformed plants leads to improvement of salt and drought tolerance of transformed plants was found during experiments. In conditions of potassium deficiency or salt stress the plants with elevated OsTPKa expression level exhibit better growth rates, decreasing of sodium accumulation in plant tissues.

Key words: OsTPKa overexpression, *Oriza sativa*, salt stress, drought stress, potassium deficiency, potassium vacuolar channels.

In conditions of continuous world population growth the one of the major task of mankind is to reduce water scarcity and land management optimization for agricultural use. The high demand for food in the future only increase. Taking into account these circumstances, the food production must increase by increasing the crop productivity and employment of marginal lands for the agricultural development [1, 2]. The one of possible way to increase agricultural productivity is growing crops on soils affected by salinity [3]. The salt toxicity is the one of the main factor limiting the productivity of agricultural crops. The creation of tolerant plants to high salinity and water stress could significantly improve agricultural productivity, particularly in deserted areas and lands that suffered salinization through irrigation.

Rice is the one of most important agricultural crops. It should be noted that it is very sensitive to salinity. The yield losses occur due to excessive salinity or water shortage. In this context, improving the salt and drought tolerance of this crop is very important for the future agricultural development.

There are several mechanisms of drought and salt tolerance improvement. One of the crucial mechanism to achieve this goal is to maintain high cytosolic concentrations of K^+ to Na^+ [4, 5]. In order to increase the K^+ content in the cytosol, the K^+ homeostasis in plants should be improved. The one of possible way to improve the K^+ homeostasis is increasing the K^+ accumulation level in vacuoles. The application of proteins responsible for K^+ transport in cell could increase the intracellular K^+ content. It worth to mention the potassium channels of TPK family.

The TPK-channels are involved in a wide range of physiological processes. They are responsible for the K^+ homeostasis, turgor pressure generation. The TPKs are participated in many types of plant response to abiotic stresses. For example, it was shown that AtTPK1 is involved in the stomata movement [6]. NtTPK1a is sensitive to the action of spermidine and spermine, and the level of gene transcripts of this channel increased by hyperosmotic conditions [7]. It was shown that gene expression of poplar TPK-channel in the tobacco cells could increase their resistance to salt stress [8]. Rice genome encodes three different isoforms TPK channels [9]. The rice TPK channels (OsTPKa and OsTPKb) exhibit different vacuolar localization. OsTPKa is targeted to the tonoplast of central lytic vacuole. OsTPKb is localized on tonoplast of protein storage vacuoles [10]. In order to increase the salt- and drought tolerance of rice plants, we have increased the level of OsTPKa gene expression.

Materials and Methods

Plant material and growing conditions. Mature seeds of japonica rice cultivar Nipponbare were used for plant transformation and all further experiments. Rice seeds were germinated in sterile conditions, at 28 °C, 100% relative humidity and kept in darkness for five days. Seedlings produced from the seeds were then transferred to a hydroponic nutrient solution (1.25 mM KNO_3; 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$; 42.5 μ MFe-EDTA; 0.625 mM KH_2PO_4; 0.16 μM $CuSO_4$; 0.38 μM $ZnSO_4$; 1.8 μM $MnSO_4$; 45 μM H_3BO_3; 0.015 μM $(NH_4)_2MO_7O_{24}$; 0.01 μM $CoCl_2$, pH 5.5–6.0). Every 7 days changed to fresh medium. 3 weeks after germination of the plants exposed to different Na^+, K^+ and water stress. Non-stressed plants were grown in parallel and harvested at the same time and served as a control. To measure the growth rate and the content of Na^+ and K^+ seedlings under 4 weeks cultivated another 2 weeks in the control and experimental media. In order to model salt stress conditions the NaCl to final concentration 50 мM was added. For the modeling of K^+ free environment, the KNO_3 and KH_2PO_4 were replaced by the corresponding salts with Na^+. The water stress was achieved by addition to the liquid nutrient solution polyethylene glycol 4000 (PEG 4000, Fluka, Switzerland) to final concentration 20%.

OsTPKa cloning and transgenic rice plants generation. OsTPKa sequence was found in EMBL GenBank accession number: OsTPKa NM_001057833. The OsTPKa cDNA clone was received from Rice Genome Resource Center — RGRC, Japan. The coding sequence of OsTPKa was cloned into the binary vector pGreen0029 (http://www.pgreen.ac.uk). The coding sequence OsTPKa was amplified by PCR with primers: OsTPKaXhoI_for GCCTCGAGATGGATGACAACAGCATT, OsTPKaSmaI_rev GCCCCGGGCTGAGCAGAT TGTGCTAG. PCR was performed in a 50 ml reaction mixture containing 1xPhusion HF PCR buffer, 200 mM dNTP, 3% DMSO, 1 ml Phusion polymerase (Finzymes, Espoo, Finland), 20 ng of DNA. The PCR amplification profile had the following parameters: 98 °C 30 s; 36 cycles of 98 s 10 °C, 72 °C 30 s; 72 °C 10 min. The PCR amplified OsTPKa-fragment was inserted into the 35S-CaMV promoter cassette plasmid pART7 [11] by restriction enzymes *XhoI* and *SmaI* (NEB) and ligation T4-DNA ligase (NEB, Hitchin, UK). The promoter cassette containing the coding sequence of OsTPKa was cloned into the binary vector pGreen0029, carrying the resistance gene to hygromycin A, using restriction enzyme NotI and ligation T4-DNA ligase. Agrobacterial transformation of rice was carried out according to the procedure described in [12]. The seeds (T1) from independent T0-lines were germinated on medium containing hygromycin A. The plants resistant to hygromycin A, were selected for further experiments. All hygromycin A resistant plants were analyzed by PCR using primer pair to the promoter sequence and OsTPKa: 35S_for GCATGGGGATGAGGTTTTTA and OsTPKa_ rev CTGAGCAGATTGTGCTAG. Each PCR was conducted in 50 ml of reaction mixture containing 1xTaq PCR buffer, 200 mM dNTP, 0.5 ml of Taq-polymerase (Promega, USA) and 200 ng of genomic DNA. PCR amplification program had the following parameters: 4 min 95 °C; 40 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 60 s; 72 °C 10 min. The plant lines that have passed through embryogenesis with sensitivity to hygromycin A were used as a control.

The genomic DNA extraction from plants. The total DNA was extracted from rice and barley plants according to the CTAB method with some modification [13, 14]. Plant material up to 100 mg was ground to fine powder in liquid nitrogen. Ground tissues were quickly mixed with pre-warmed 450 μl of CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCL (pH 8) and 20 mM Na-EDTA) and incubated at 65 °C for 50 min. After vortexing the mixture, 300 μl of chloroform:isoamylalcohol solution (24:1) was added. The mixture was vigorously shaken and centrifuged for 5 min in microfuge. The top aqueous layer was transferred to clean sterilized eppendorf tubes and DNA was precipitated by adding 2 volumes of 96% ethanol and 4% 3 M NaAc (pH 5.2). The mixture was vortexed and left at room temperature for 30 minutes to precipitate the DNA. The mixture was then centrifuged for 10 min at 13000 rpm to obtain the DNA pellet. Finally, the pellet was rinsed in 70% ethanol, dried for 10 min and resuspended in 100 μl TE buffer.

Transcriptional analysis of plants. Total RNA was isolated from rice tissues of using Qiagen RNeasy kit (Qiagen, Germany) according to the manufacturer’s instructions. 1 mg of total RNA was used for subsequent cDNA synthesis. The cDNA synthesis was done by application of SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to manufacturer's instructions. The level of OsTPKa expression was measured by Real-time PCR. The SYBR Green master mix (Applied Biosystems, USA) and corresponding device ABI-7900 sequence detection system (Applied Biosystems, USA) were used to perform Real-time PCR. There are specific primer pairs to OsTPKa: OsTPK1aSybr_for CAAGGCCCCCCTGAAAAG and OsTPK1aSybr_rev CTTGGCCTCATCTCCTTG AATAA; and to OsEF1a (elongation factor 1α): OsEFasybr_for CAAGGCCCGTTACGA TGAAAT and OsEFasybr_rev GGTTGTAGC CGACCTTCTTCAG were used in Real-time PCR. The expression level of housekeeping OsEF1a gene was taken as control [13].

Estimation of plant relative growth rate. The transformed and control plants aged 3 weeks were transferred on different cultivation mediums for 2 weeks. We used 5 to 6 plants for each line and for each type of stress. The relative growth rate determined in accordance to [15]. The calculation of relative growth rate was conducted according to following formula: (ln (FW2) -ln (FW1) / (t2-t1), where FW — fresh eight of plant tissue, and t1 and t2 — start and end times of growth period (14 days). The 4 plants were weighted for the each t1 and t2 times for each type of stress. No significant differences between control and transformed plants growed on standard hydroponic nutrient solution were found. The experiments had three biological replications. The data were presented by average values and ± standard deviation (SD).

Determination of Na^+ content in plant tissues. After two weeks of plant cultivation on different types of liquid medium, the 4 plants for each type of stress and control conditions were collected. Consequently, the plants were divided into stem and root parts. The roots were washed twice in ice cold solution of 20 mM $CaCl_2$ for 10 min. The dry and fresh weights of plant tissue samples were determined. Dried samples were grinded. Ion extraction was performed in 5 ml of 20 mM $CaCl_2$ for 24 hours [16, 17]. The concentration of Na + was evaluated by flame photometry (Sherwood Scientific Ltd, UK). Experiments were repeated three times. The data were presented by average values and + standard deviation (SD).

Results and Discussion

During the experiments the plant transformation by callus induction in mature grains of rice was performed. The transformants were selected by resistance to hygromycin A. After PCR analysis of

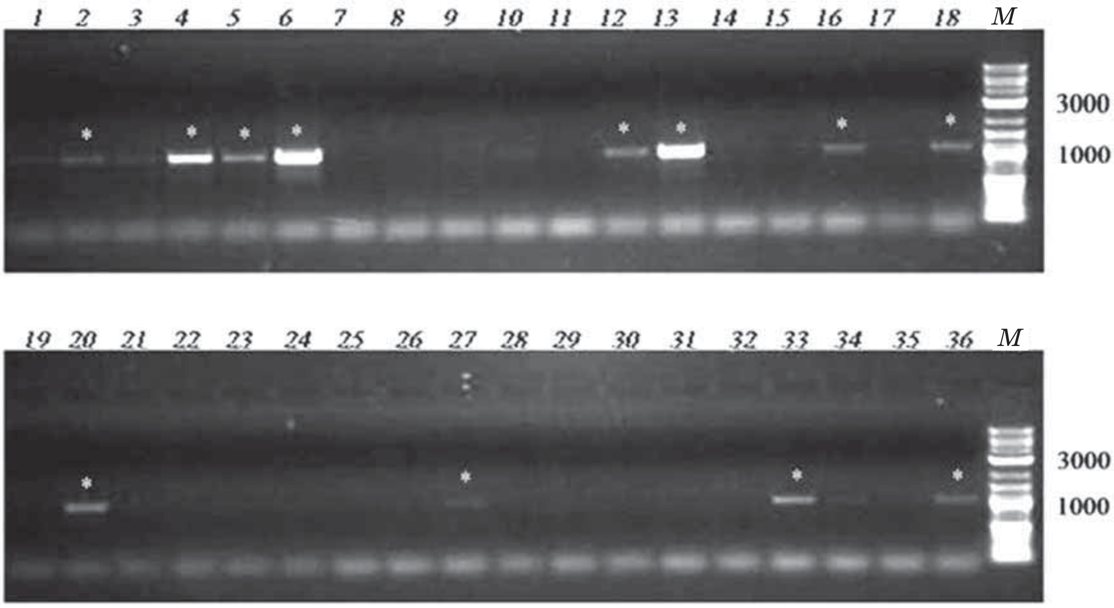


Fig. 1. **PCR analysis of primary rice transformants with primers specific to CDS of OsTPKa and CaMV 35S promoter:**

1, *19* — negative control,water;
2–18, *20–36* — genomic DNA from independent transformed rice lines;
M — molecular marker. The lines marked by asterisks were selected for further analysis

hygromycin A resistant transformants the 12 independent lines were selected (Fig. 1). In order to evaluate the functionality of the obtained transgenic plants the OsTPKa expression analysis by RT Real-time PCR was conducted. The expression analysis of transgenic plants, the 8 plant lines exhibited the OsTPKa expression level significantly higher than in control plants (Fig. 2). The level of OsTPKa transcripts in transformed plants was significantly higher than in control plants (40 to 100 times). In order to conduct further research the 4 plant lines (2, 4, 6, 8) with relatively similar levels of OsTPKa expression were chosen.

Together with control plants the selected transgenic plants were transferred on liquid hydroponic mediums for the modeling of salt stress by addition of NaCl (50 mM), water stress by addition of PEG 4000 to a final concentration of 20% , creating K^+ deficiency. The experimental data demonstrate that all these tested types of stresses, significantly reduce the growth rate of both control and transgenic plants (Fig. 3). Whatever, analysis of the relative growth rate of tested shows that elevation of OsTPKa expression level could improves the plant growth in high NaCl conditions (Fig. 3). Interestingly, it was almost no differences in relative growth rates for transgenic and control plants cultivated on K^+ free hydroponic medium (Fig. 3). A small variation that was observed for this type of stress was not significant. The transgenic plants cultivated in water stress conditions (20% PEG 4000) exhibit greater tolerance in comparison with control line. Moreover, the difference in the relative growth rates in plants overexpressing OsTPKa was higher than in experiments with salt stress conditions (Fig. 3).

The main aim of our experiments was to investigate how the overexpression of gene encoding OsTPKa potassium channel can affect Na^+ accumulation and K^+ homeostasis in plants. The Na^+ content analysis in plant demonstrates reduction of accumulation level of this cytotoxic ion in tissues of OsTPKa overexpressing plants in comparison control (Fig. 4). The Na^+ content of was measured as for aerial parts as well as for root system. According to our observation the shoot Na^+ content in transgenic plants was significantly lower to controls (Fig. 4, *A*). The similar pattern was observed for the root Na^+ content. In most cases the tissue Na^+ content was lower in OsTPKa overexpressing plants in comparison with controls. It worth to note, that relatively high Na^+ accumulation level was detected in root tissues of transgenic plants cultivated on K^+ free medium (Fig. 4, *B*). This imbalance in the Na^+ contents between roots and shoots can be explained by involving compensatory mechanisms of plants to maintain adequate osmotic status. There are several works that show the Na^+ accumulation in plant tissues in K^+ deficiency conditions [17, 18]. Moreover, it should also be noted that the Na^+ accumulation was observed in root system, but not in the shoots, where the active photosynthesis. The

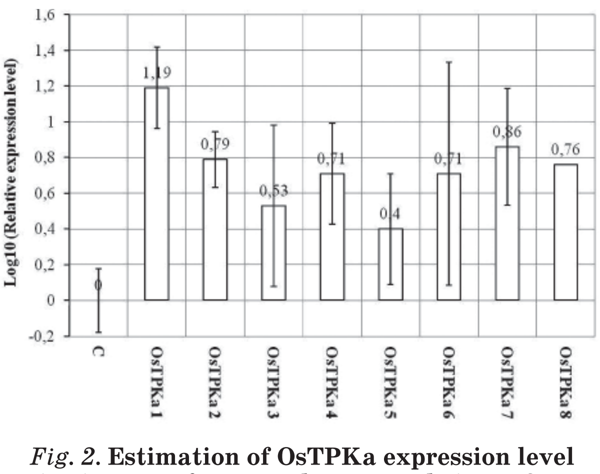


Fig. 2. **Estimation of OsTPKa expression level in rice transformants by RT Real-time PCR:**
C — nontransformed control; OsTPKa *1, 2, 3, 4, 5, 6, 7, 8* — transformed rice lines

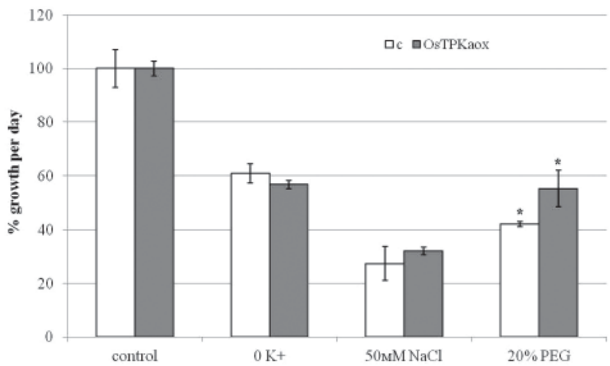
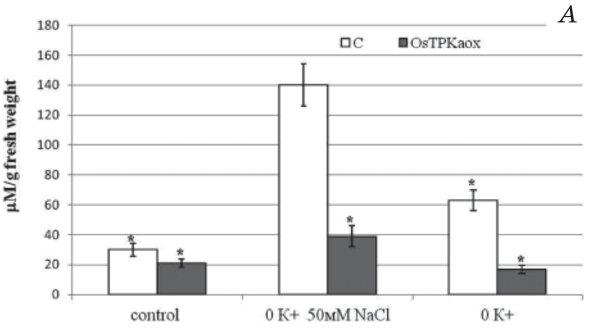


Fig. 3. **Estimation of relative growth rate of trasformants and control plants in different stress conditions:**
C — nontransformed control plants;
OsTPKaox — OsTPKa overexpressing plants.
Control — standard hydroponic nutrient solution;
0 K^+ — K^+ free nutrient solution (K^+ deficiency);
50 мM NaCl — nutrient solution with additional 50 мM NaCl (salt stress);
20% PEG — nutrient solution with additional PEG 4000 to final concentration 20% (drought stress).
Here and further:
* — $P < 0{,}05$ in comparison with control. All the experiments were subjected to unpaired two-tailed *t*-tests to identify significance at the $P < 0.05$ level



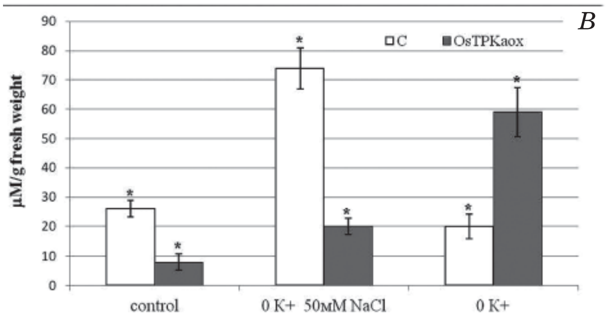


Fig. 4. **Estimation of Na^+ content in tissues of transformed and control plants subjected to salt stress and K^+ deficiency:**
C — nontransformed control plants;
OsTPKaox — OsTPKa overexpressing plants.
Control — standard hydroponic nutrient solution;
0 K^+ 50 мM NaCl — K^+ free nutrient solution with additional 50 мM NaCl (K^+ deficiency and salt stress);
0 K^+ — K^+ free nutrient solution (K^+ deficiency):
A — Na^+ content in shoots;
B — Na^+ content in roots

excessive levels of this cytotoxic ion have negative impact on this important biosynthetic process. Therefore, there is a redistribution of K^+ to active biosynthetic tissues takes place. Moreover, the Na^+ ions will be accumulated in the less demanding and metabolically active plant tissues, particularly in the roots (Fig. 4).

Thus, it was found that overexpression of the gene encoding the potassium channel OsTPKa in rice, can significantly increase salt and dought tolerance of plants. It was shown that OsTPKa overexpressing plants have better growth rates in conditions of salt and drought stress. The elevation of OsTPKa gene expression improves the K^+ homeostasis plants. It is very likely that the transgenic plant tissues contain more K^+ ions, than control. Regarding that, these plants have improved K^+ homeostasis and therefore accumulate in their tissues significantly less amount of cytotoxic Na^+ in order to overcome the osmotic shock. It is known, that osmotic shock is associated with salt and drought stress.

Thus, the approach of high K^+ cytoplasmic concentrations maintaining to Na^+ in plant tissues is a promising direction for the future applications in plant biotechnology. Our data indicate that overexpression of genes encoding TPK potassium channels could improve the K^+ homeostasis of plants and tolerance to high salt concentration and water deficiency.

The generated transgenic plant lines could be used for further breeding or biotechnological improvement of rice varieties for cultivation in areas affected by water shortage and excessive salinity.

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ПІДВИЩЕННЯ СТІЙКОСТІ РОСЛИН РИСУ ДО СОЛЬОВОГО ТА ВОДНОГО СТРЕСУ НАДЕКСПРЕСІЄЮ ГЕНА, ЩО КОДУЄ КАЛІЄВИЙ КАНАЛ OsTPKa

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Метою роботи було підвищення соле- та посухостійкості рослин рису за допомогою надекспресії гена одного із TPK-каналів, а саме OsTPKa. Цього було досягнено методом агробактеріальної трансформації рослин геном, що кодує калієвий двопоровий канал OsTPKa. У ході проведення експериментів виявлено, що підвищення рівня експресії гена цього каналу в трансформованих рослинах рису поліпшує показники стійкості до високих концентрацій солей, водного стресу та дефіциту K^+. Рослини, які мали підвищений рівень експресії OsTPKa, демонстрували кращий рівень росту, зменшення накопичення іонів Na^+ у своїх тканинах за умов сольового стресу та дефіциту K^+.

Ключові слова: надекспресія гена, що кодує калієвий канал OsTPKa, *Oriza sativa*, сольовий стрес, водний стрес, дефіцит калію, вакуолярні калієві канали.

ПОВЫШЕНИЕ УСТОЙЧИВОСТИ РАСТЕНИЙ РИСА К СОЛЕВОМУ И ВОДНОМУ СТРЕССУ С ПОМОЩЬЮ НАДЭКСПРЕССИИ ГЕНА, КОДИРУЮЩЕГО КАЛИЕВЫЙ КАНАЛ OsTPKa

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Целью работы было повышение соле- и засухоустойчивости растений риса с помощью надэкспресии гена одного из TPK-каналов, а именно OsTPKa. Это было достигнуто методом агробактериальной трансформации растений геном, кодирующим калиевый двухпоровый канал OsTPKa. В ходе проведения экспериментов было установлено, что повышение уровня экспрессии гена этого канала в трансформированных растениях улучшает показатели устойчивости к высоким концентрациям солей, водному стрессу и дефициту K^+. Растения с повышенным уровнем экспрессии OsTPKa демонстрировали лучшие показатели роста, уменьшение накопления Na^+ в своих тканях в условиях солевого стресса и дефицита K^+.

Ключевые слова: надэкспрессия гена, кодирующего калиевый канал OsTPKa, *Oriza sativa*, солевой стресс, водный стресс, дефицит калия, вакуолярные калиевые каналы.