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PHENOTYPIC ANALYSIS OF OsTPKb LOSS OF FUNCTION MUTANT RICE LINES

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The results of screen and analysis of two OsTPKb rice mutant lines were described. The phenotypes and growth rate level of homozygous mutant plants of both rice lines were estimated. The electron microscopy of aleurone layer from forming seeds was performed. The OsTPKb mutant plants demonstrate lower growth rate in comparison with wild type plants. The loss of function OsTPKb mutations invariably led to (semi)sterile rice plants. The functional disruption of OsTPKb channel has negative impact on plant growth and development and might completely change the cell morphology of aleurone layer.

Key words: OsTPKb, TPK-channels, seed formation, potassium homeostasis, rice, *Oryza sativa*, mutant analysis.

Potassium (K^+) is one of the main elements of plant mineral nutrition. This element has primary importance for normal plant growth and development. The main reservoir of intracellular K^+ vacuole is a and tonoplast K^+ channels of the TPK (Two Pore K) family are main players in cellular K^+ homeostasis. The functions and physiological role of TPK channels are diverse. In addition to maintain of K^+ homeostasis, the TPK channels are involved in turgor regulation and responses to various abiotic stresses [1, 2].

Most of the TPK channel members are localized in the tonoplast of lytic vacuole [1, 3]. Besides central lytic vacuole plant cells might have another type of vacuoles. Recently it was shown that the two rice isoforms of TPK channel TPK family (OsTPKa and OsTPKb) have different vacuolar localization [4]. In contrast with OsTPKa localization in tonoplast of central lytic vacuole, OsTPKb is localized in small protein storage vacuoles. Rice genome encodes at least three different isoforms two-pore TPK channels, OsTPKa, OsTPKb and OsTPKc. Interestingly, the OsTPKa and OsTPKb sequences are very similar to each other (63% identity) [1]. However biophysical characteristics and membrane localization of these two proteins are significantly different [4]. It worth to note, that the protein storage vacuoles may comprise several internal compartments which have no analogues in animals and yeast [5, 6]. The protein storage vacuoles have been found in all cell types of plant tissues. But they are particularly numerous in tissues of the reproductive organs and seeds. Unlike lytic vacuoles responsible for degradation and lysis, protein storage vacuoles are involved in storage of important minerals and other compounds in plant cell [1, 5]. Protein storage vacuoles play an important role in creating the reserves of nutrients and minerals in plant seeds. The cells aleurone layer of grains is extremely rich by this type of vacuoles [5]. It should be also noted, that consumption of plant grains or seeds is basically protein storage vacuole "diet" [6]. As was noted earlier, OsTPKb is a specialized K^+ channel of protein storage vacuole membrane. It is very likely, that this protein is responsible for the accumulation of K^+ in seeds. Thus it has big influence on protein storage vacuole formation and seed development. It is worth to mention, that rice is one of the most important world agricultural crop. Therefore, the study of mechanisms of seeds formation, storage and accumulation of pivotal mineral nutrients in the seeds is extremely important.

The aim of our study was to estimate the role of OsTPKb channel in the formation of

seeds and the growth and development of plants by screening and phenotypic analysis of OsTPKb rice mutants.

Materials and Methods

The plant material and identification of OsTPKb rice mutants. Rice mutant lines were identified and obtained through the RiceGE database (http://signal.salk.edu) [7]. By application online analysis of *OsTPKb* gene patterns and appropriate databases for selection of mutant lines the two *OsTPKb* mutant lines were selected, retransposon insertion line Tos 17 (NF6453) from the Rice Genome Resource Center RGRC (Japan, https://tos.nias.affrc.go.jp) and T-DNA insertion line of (PFG_2D-41178.R) from POSTECH (Pohang University of Science and Technology, South Korea, http://www.postech.ac.kr/life/pfg) (Fig. 1) [8]. The Nipponbare variety of rice plants was used as control wild type (WT) plants.

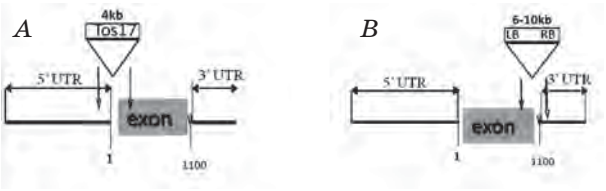


Fig. 1. **The scheme of insertional mutations in two different rice mutant lines:**
A — NF6453 (*Tos17*) line;
B — PFG_2D-41178.R (Т-ДНК) line

Rice seeds were germinated under sterile conditions at 28 °C, 100% relative humidity in enclosed plastic containers that do not reflect light. After 7 days of germination, the young seedlings replaced into liquid culture medium (1.25 mM KNO_3; 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$; 42.5 μM Fe-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 the cultivation medium was replaced by freshly made nutrient solution.

Isolation of plant genomic DNA. The total DNA was extracted from rice plants using CTAB method with some modifications. 100 mg of plant material was homogenize to fine powder by porcelain mortar and pestle in liquid nitrogen. To 100 mg of homogenized plant tissue the 450 ml pre-warmed CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8) and 20 mM Na-EDTA) was added. The plant tissue was incubated at 65 °C for 50 min. After careful mixing by Vortex the 300 ml of chloroform: isoamyl alcohol (24: 1) was added. The solution was mixed and centrifuged for 5 min at 12 000g. The upper aqueous phase were transferred into a new microcentrifuge tube. The DNA fraction was precipitated by addition of 96% ethanol and 4% of 3 M NaAc (pH 5.2). The mixture was mixed by Vortex and incubated for 30 min at room temperature. The mixture was centrifuged for 10 min at 12 000g. The resulting precipitate was washed in 70% ethanol and centrifuged again for 20 min. Dried for 10 minutes pellet was resuspended in 100 ul TE-buffer.

Genotype identification rice mutant lines by PCR. The genotype type identification of rice *Tos17* insertion line NF6453 was conducted by PCR using primers specific to the coding sequence of the *OsTPKb* gene: OsTPK1b(c)_for ATGGCGGCCCTCGACCAACA and OsTPKb(c)_rev ACGCAGGGAAGGCGGCGGGT, and primer combination OsTPKb(c)_rev with Tos17-specific primer Tos17-1_rev ATTGTTAGGTTGCAAGTTAGTTAAGA. Homozygous plants were selected for further analysis. The PCR was performed in a 50 ml reaction mixture containing 1xTaq PCR buffer, 200 mM dNTPs and Taq-polymerase 0,5 ml (Promega, USA) with 50 ng of genomic DNA. The amplification profile was: 95 °C 4 min; 40 cycles: 95 °C 30 s; 54 °C 30 s; 72 °C 60 s; 72 °C 10 min.

The analysis of insertional *OsTPKb* mutant line PFG_2D-41178.R was conducted by PCR as well. In order to identify the plant genotypes (wild-type (Wt), homozygous by T-DNA insert (Hm) and heterozygous (Hz)) the next combinations of primers for PCR were applied: PFG_for AAACTTAAAGCGAGTACGGAGG and PFG_rev CCCTCTACTTCTGCGTCGTC, specific to flanked T-DNA regions (http://signal.salk.edu), and the combination of primers and PFG_rev OsTPKb (s) _rev. PCR was performed as follows: 50 ml of the reaction mixture included 1xTaq PCR buffer, 200 mM dNTPs and Taq-polymerase 0,5 ml (Promega, USA) with 50 ng of genomic DNA. The amplification profile was: 95 °C 4 min; 40 cycles: 95 °C 30 s; 54 °C 30 s; 72 °C 60 s; 72 °C 10 min.

Gene expression analysis in OsTPKb mutant rice lines by RT-PCR. The total RNA was isolated from rice green tissues by RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. 1 microgram of total RNA was used for cDNA synthesis by

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). PCR reaction was performed using primers specific to *OsTPKb*: OsTPKb_for GGTGCGCGCCATGGAGATGA and OsTPKb_rev GAGTGATCGGCGTCGAGGTT. The another primer pair was specific to rice *actin 1* (*OsAct1*): OsAct1_for CGCTTCCTCATGCTATCCTC OsAct1_for GCTAGGAGCAAGGGAAGTGA. PCR amplification program had the following parameters: 95 °C 4 min; 40 cycles: 95 °C 30 s; 54 °C 30 s; 72 °C 30 s; 72 °C 4 min. The gene expression level of *actin 1* was taken as constitutive [9].

Aleurone layer electron microscopy of forming rice seeds. The morphology of aleurone layer of forming seed tissue was analyzed by electron microscopy. The 15 days old immature rice seeds were collected from rice plants of wild type and *OsTPKb* mutant rice lines. From collected immature seeds the aleurone layers were isolated and fixed in 4% paraformaldehyde, 0.2% glutaraldehyde and 50 mM sodium phosphate buffer (pH 7.2) at 4 °C. After washing 3 times with a solution of 50 mM sodium phosphate buffer (pH 7.2), aleurone layer tissues subsequently were fixed and stained by osmium tetroxide (1% OsO_4, 50 mM sodium phosphate buffer (pH 7.3)) for 1.5 hours at 4oC. Subsequently the samples were subjected to gradual series of dehydration by incubation in ethanol with different concentrations, 70, 80, 90, 95 and 100%. After dehydration sample were immersed into a special white resin (LR white Resin (London Resin Co. UK) for further section preparation (70 nm) using ultramicrotome Leica UCT (Germany). The resulted sections were applied on copper grid for electron microscopy and stained for 10 minutes uranyl acetate solution of 4% and lead citrate. Microscopic analysis was carried out at 60-80 kV transmission electron microscope TECNAI G2 12 (FEI, USA).

Results and Discussion

In order to determine the functional role of OsTPKb two-pore channel in rice, the search and identification of OsTPKb mutant lines by application of several online rice genome resources was conducted. The two different mutant lines were selected. These two selected mutant lines are based on insertion of retransposon element Tos17 or T-DNA (Fig. 1). The seeds of these rice mutant lines were ordered in two different independent centers for rice genome research. The obtained seeds were germinated and DNA was isolated for identification of homozygous lines and further application in experiments. Three homozygous plants of PFG_2D-41178.R (T-DNA insertion) lines and 2 plants of NF6453 (Tos 17) line were selected after PCR analysis (Fig. 2). After confirmation of mutant homozygosity, the analysis of *OsTPKb* gene expression in homozygous mutant plants were tested by RT-PCR. No *OsTPKb* transcripts were detected in homozygous plants for both mutant strains (Fig. 3). Selected by PCR and RT-PCR analysis plants were transferred to hydroponic medium for further cultivation. It was observed during the cultivation period, that lack of *OsTPKb* gene products in plants has significant negative impact on their growth and development. Plants of mutant lines had much lower growth rate in comparison with heterozygous and wild-type plants (Fig. 4). Moreover, according to our observations, mutations in the *OsTPKb* gene led to semisterility of adult plants.

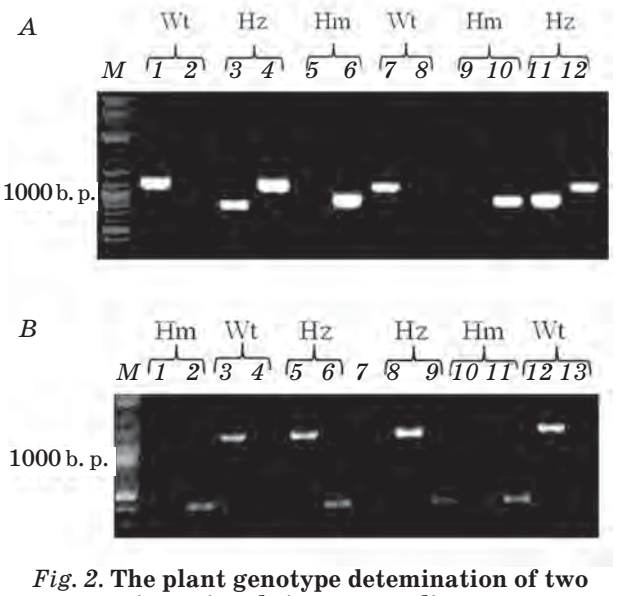


Fig. 2. **The plant genotype detemination of two insertional rice mutant lines:**

Hm — homozygous; Hz — heterozygous; Wt — wild type;

A — NF6453 (*Tos17*) line: *1, 3, 5, 7, 9, 11* — PCR with primers OsTPKb(c)_for and OsTPKb(c)_rev. *2, 4, 6, 8, 10, 12* — PCR with primers OsTPKb(c)_rev and Tos17-1_rev. Groups *1* and *2*; *3* and *4*; *5* and *6*; *7* and *8*; *9* and *10*; *11* and *12* — DNA samples from single plants;

B — PFG_2D-41178.R (T-DNA) line: *1, 3, 5, 8, 10, 12* — PCR with primers PFG_for and PFG_rev. *2, 4, 6, 9, 11, 13* — PCR with primers PFG_rev and OsTPKb(c)_rev. Groups *1* and *2*; *3* and *4*; *5* and *6*; *8* and *9*; *10* and *11*; *12* and *13* — DNA samples from single plants

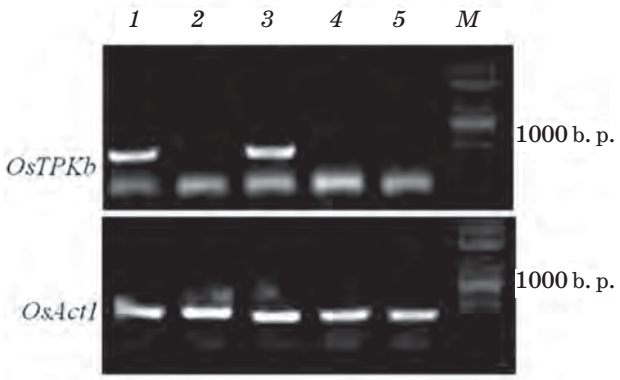
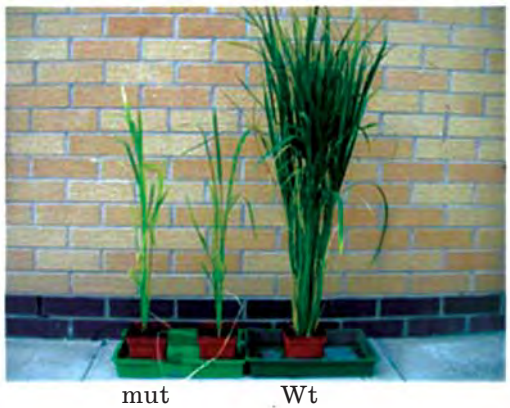


Fig. 3. **RT-PCR analysis of *OsTPKb* transcript level in wild type plants (Wt), heterozygous (Hz) and homozygous plants (Hm) of NF6453 (*Tos17*) and PFG_2D-41178.R (T-ДНК) mutant lines:**
OsTPKb — PCR products of OsTPKb transcripts;
OsAct1- PCR products of *OsActin 1* transcripts.
1 — Wt; *2* — Hm (PFG_2D-41178.R); *3* — Hz _2D-41178.R); *4* — Hm (PFG_2D-41178.R); *5* — Hm (NF6453)



mut Wt
NF6453 (*Tos17*)

Wt mut
PFG_2D-41178.R (T-DNA)

Fig. 4. **The phenotypes of wild type plants (Wt) and *OsTPKb* rice mutant lines (mut)**

One of the main problem we have faced during study the OsTPKb channel functional role was obtaining viable seeds. The seeds that were formed in the homozygous mutant plants had weak ability to germinate, were mostly "empty" or with other morphological disorders.

It is known, that the OsTPKb is maine K^+ channel of protein storage vacuoles [1, 5, 6]. The cells of seed tissue, in particular aleurone layer, are exceptionally enriched in protein vacuoles. Protein storage vacuoles have an important function of storing nutrients and pivotal mineral elements in the seeds [2, 5]. Perhaps this unique function of these plant cell organelles is crucial for the successful formation of plant seed. Therefore it was decided to check the aleurone layer morphology of forming rice seeds in *OsTPKb* mutant plants. According to results of electron microscopy observation the significant disorders of aleurone layer and adjacent tissues cell morphology were detected in rice seeds of mutant rice lines (Fig. 5). The cells of aleurone layer of mutant plants did not have the protein storage vacuoles and other cellular structures involved in storage of mineral elements and nutrients (Fig. 5). Additionally, as result of theses abnormalities, the rice seeds were unable to form a complete cell set of aleurone layer and possibly had negative impact on neighboring embryonic cells.

Thus, the function of OsTPKb K^+ channel is very important and necessary for the full development and reproduction of plants. According to our observations (unpublished data), the overexpression of *OsTPKb* gene leads to increased plant resistance to certain types of abiotic stresses such as salinity and osmotic shock. Our data prove the big importance of protein storage vacuoles for cell osmoregulation. The OsTPKb could be a key player to maintain this function. The data analysis from the Genevestigator (https://genevestigator.com/gv/) indicates that the level of gene transcripts are rised under condition of salt and osmotic stress [1]. Moreover, according to data from Genevestigator, the *OsTPKb* highest gene expression level is observed in the early stages of germination, where the role of protein storage vacuole is extremely important. It worth to mention that enrichment by K^+ the cytoplasm of embryonic cells triggers the activation of various metabolic processes during germination. The role of OsTPKb is extremely important for development and conduction of these processes. It is well known,

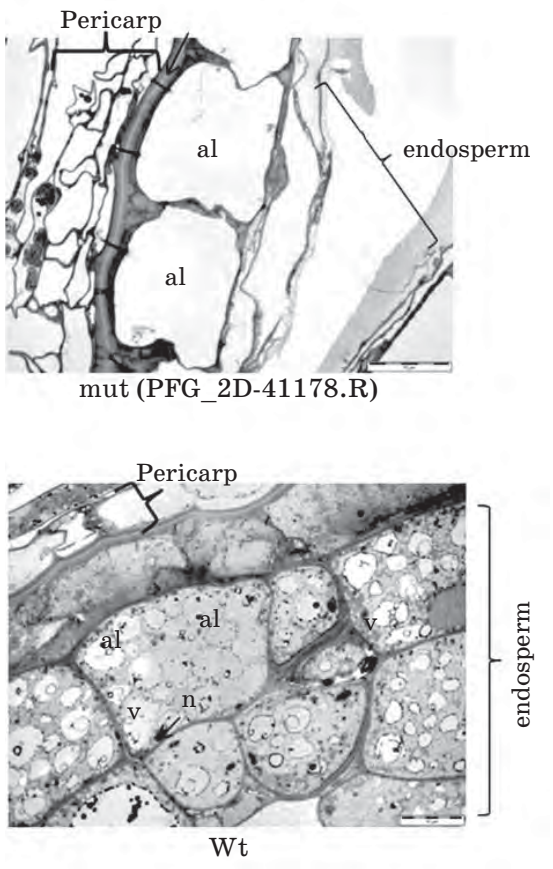


Fig. 5. **The aleurone layer electron microscopy of immature seeds from wild type plants (Wt) and *OsTPKb* mutant line (mut):**
al — aleurone layer cells; v — vacuoles; n — nuclei

that K^+ is one of the most important mineral elements for plants. This element is involved in more than 200 enzymatic reactions in cell. TPK channels are the key elements of maintaining K^+ homeostasis in the plant. Therefore, the possible reduction of K^+ content in rice *OsTPKb* mutant, led to significant inhibition of plant growth and development. The unique location of this channel in protein storage vacuoles, makes it extremely important for proper formation and adequate functioning of seeds.

Thus, according to isolation and analysis of rice *OsTPKb* mutants, the homozygous plants of two mutant lines were selected. These homozygous mutant plants exhibited no expression of *OsTPKb* gene. Morphological analysis of the homozygous *OsTPKb* mutant plants indicates important role of OsTPKb K^+ channel in plant growth and development. It was shown that mutations in *OsTPKb* lead to semisterility of plants and disorders in seed formation. The analysis of immature seed morphology by electron microscopy have shown abnormalities of aleurone layer in mutant plants. The aleurone layer cells of mutant lines had degraded structure without protein storage vacuoles.

Thus the application of mutant screen and analysis of phenotypes revealed the important role of OsTPKb potassium channel for the growth and development of plants and seed formation.

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ФЕНОТИПОВИЙ АНАЛІЗ МУТАНТНИХ ЛІНІЙ РИСУ ІЗ ВТРАЧЕНОЮ ФУНКЦІЄЮ K^+ КАНАЛУ OsTPKb

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Наведено результати пошуку та аналізу двох мутантних ліній рису за геном, що кодує калієвий двопоровий канал OsTPKb. Було оцінено відносний рівень приросту та фенотип гомозиготних рослин обох мутантних ліній. Проведено аналіз морфології алейронового шару незрілого насіння рису за допомогою електронної мікроскопії. Мутантні за геном OsTPKb рослини рису характеризуються набагато меншими темпами приросту біомаси порівняно з рослинами дикого типу. Цим мутантам притаманні часткова стерильність та порушення процесів формування насіння. Втрата функції цього каналу в рослин негативно впливає на формування насіння та змінює морфологію клітин алейронового шару зернівок.

Ключові слова: OsTPKb, канали родини ТРК, формування насіння, гомеостаз калію, рис, *Oryza sativa*, аналіз мутантів.

ФЕНОТИПИЧЕСКИЙ АНАЛИЗ МУТАНТНЫХ ЛИНИЙ РИСА С УТРАЧЕННОЙ ФУНКЦИЕЙ K^+ КАНАЛА OsTPKb

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Представлены результати поисков и анализа двух мутантных линий риса по гену, кодирующему калиевый двупоровый канал OsTPKb. Была проведена оценка относительного уровня прироста и фенотипа гомозиготных растений обеих мутантных линий. Осуществлен анализ морфологии алейронового слоя незрелых семян риса с помощью электронной микроскопии. Мутантные по гену OsTPKb растения риса характеризуются намного меньшими темпами прироста биомассы по сравнению с растениями дикого типа. Этим мутантам свойственны частичная стерильность и нарушения процессов формирования семян. Потеря функции этого канала негативно влияет на формирование семян и изменяет морфологию клеток алейронового слоя зерновок.

Ключевые слова: OsTPKb, каналы семйства ТРК, формирование семян, гомеостаз калия, рис, *Oryza sativa*, анализ мутантов.