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CHARACTERIZATION OF *RPP5CS* AND *RPOAT* FROM *RUMEX PATIENTIA* L. IN RESPONSE TO SALINITY AND ALKALINITY STRESS

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Abstract

Rumex patientia L. can respond to abiotic stresses and withstand low temperatures. Proline accumulation plays important roles under various abiotic stresses in different plants, specially salinity. P5CS and OAT of plants are key enzymes in the proline synthesis pathway. In the present study, the *RpP5cs* and *RpOat* genes of the proline synthesis pathway in *R. patientia* L. were cloned with full-length cDNAs of 2178 bp and 1401 bp. The expression of *RpP5cs* and *RpOat* genes were higher in Russian Щавель Чемпион than Chinese *R. patientia* × *R. tianschanicus* cv. Rumex under salinity and alkalinity stress over 0 - 24 hrs. These results could provide insights into sequence structure and differential tolerance in many plants.

Introduction

Rumex patientia L. (2n = 10) is a vegetable grown in some countries including Turkey and India (Qi *et al.* 2012). *R. patientia* can respond to temperature and various stresses (Namgail *et al.* 2007, Singh *et al.* 2013). The transcriptome of *Rumex patientia* L. was analyzed and cultivated in cold regions during cold stress (Liu *et al.* 2017). Variable environments could cause negative effects on growth and productivity, especially salinity and alkalinity stress (Li *et al.* 2015). Proline is an important osmotic regulator when plants respond to environment change. Usually plants are influenced to external stresses (Peng *et al.* 2019, 2020), all kinds of osmoregulation substances, such as proline, betaine, and monosaccharides which will accumulate to deal with the stress through osmotic adjustment (Serrano 1996). Proline accumulation is influenced by drought, salinity, metals, and biotic stresses (Kavi *et al.* 2005, Wang *et al.* 2007). In plants, the proline synthesis pathway can be divided in two: glutamate and ornithine pathway (Fig. 1) (Adams and Frank 1980, Delauney *et al.* 1993, Kavi *et al.* 2005). In the glutamate pathway, proline is generated by two reduction reactions of glutamate catalyzed by the enzymes △-pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). P5CS (EC2.7.2.11) catalyzes glutamate via the phosphorylation process, which also reduces the product to form glutamate semi-aldehyde (GSA) (Hu *et al.* 1992). In the ornithine pathway of proline synthesis, ornithine is catalyzed to the product pyrroline-5-carboxylate via ornithine-delta-aminotransferase (OAT), the OAT(EC2.6.1.13) enzyme is present in mitochondria (Verbruggen and Hermans 2008).

P5CS is a key, bifunctional enzyme (Hu *et al.* 1992) and the rate-limiting enzyme (Kavi *et al.* 2005) for proline production via the glutamate pathway in plants. The *P5cs* gene was cloned from many species, e.g. *Arabidopsis thaliana* (Thompson *et al.* 1997), *Brassica napus* (Kubala *et al.* 2015), *Phaseolus vulgaris* (Chen *et al.* 2013), *Medicago truncatula* (Armengaud *et al.* 2004). The

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expression of *P5cs* genes is induced via various abiotic stresses, e.g., the *OsP5cs1* gene is expressed in response to salinity, drought, and cold stress (Igarashi *et al.* 1997). Over-expression of *P5cs* genes in *Arabidopsis thaliana* (Chen *et al.* 2013), wheat (Vendruscolo *et al.* 2007), and chickpea (Ghanti *et al.* 2011) resulted increased proline production and salt tolerance. The ornithine-delta-aminotransferase encoded by the delta-OAT (*Oat*) gene is the key enzyme for the synthesis of proline from the pathway using ornithine as the precursor (Kavi Kishor *et al.* 2005). Over-expression of *AtOat* in tobacco increased proline production and drought tolerance (Nancy *et al.* 2002), over-expression of *Oat* in rice increased salt tolerance (Wu *et al.* 2003), and molecular and functional analyses of OAT were shown in glutamine biosynthesis during pine germination (Canas *et al.* 2008).

Fig. 1. Plant Proline biosynthesis and degradation pathways (Kavi Kishor *et al.* 2005).

In previous studies, the *P5cs* and *Oat* genes of plants decreased in response to salinity and alkalinity stress. In the present study, *P5cs* (*RpP5cs*) and *O*at (*RpOat*) from *R. patientia* L. were cloned based on transcriptome data (SRP11657). The molecular characterization of RpP5CS and RpOAT protein sequences were predicted, e.g. conserved domain subcellular localization, three-dimensional (3D) structure, etc. The expression pattern of *RpP5cs* and *RpOat* were analyzed in response to salinity and alkalinity treatments in Щавель Чемпион (L.) and Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex over a period of 24 hrs. These results could provide insights into sequence structure and differential tolerance in many plants.

Materials and Methods

Rumex patientia seeds were collected from Russian Щавель Чемпион (L) and Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex (as control). All seeds were grown in Northeast Agricultural University with a photoperiod of 16/8 hrs at 24 ± 2°C. The *R. patientia* plants were stressed with Na_2CO_3:$NaHCO_3$ = 1: 1 at a concentration 80 mmol/l at two leaves stage. In accordance with the previous study (Liu *et al.* 2017), all plants were transferred to treatment for 0, 3, 6, 12, or 24 hrs as the experimental group. For the three replicates, 0.1 g entire seedings were extracted and quickly transferred to liquid nitrogen for 10 min. The samples were stored at -80°C.

Trace archives for *Rumex patientia L.* transcriptome data (SRP11657) were searched using the "Cross-species Mega BLAST" on the Nucleotide BLAST website, all known *P5cs* and *Oat* sequences in plants were used as BLAST queries under default parameters. The conserved domain

was predicted using the NCBI conserved domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The PCR primers for *RpP5cs* and *RpOat* genes were designed based on unigenes data presented in Table 1. The PCR conditions for the *RpP5cs* gene were 95°C 5 min. followed by 35 cycles of 95°C 30 sec., 50°C 35 sec., and 72°C 150 sec., the final extension was 72°C for 8 min. The PCR conditions for the *RpOat* gene were 95°C 5 min. followed by 35 cycles of 95°C 30 s, 53°C 35 sec, and 72°C 90 s, the final extension was 72°C 8 min. The 2178 bp PCR product for *RpP5cs* and 1401 bp PCR product for *RpOat* were then subcloned into the pEAST-T3 cloning vector (TransGen Biotech) and sequenced.

Table 1. The cloning primers of *RpP5cs* and *RpOat* genes.

Name	Sequence (from 5´ to 3´)
RpP5cs forward primer	ATGGACTCCATGGATGCGTC
RpP5cs reverse primer	TCATGCCATTTTTACTGTTGTTAAG
RpOat forward primer	ATGTTAACCAAGCGACTCTTC
RpOat reverse primer	TCAAATTTCCCGCCCAC

The physicochemical characteristics of the identified protein sequence of *RpP5cs* and *RpOat* genes, e.g., amino acids (aa) composition, molecular weight (MW), and isoelectric point (pI) were analyzed using ExPASy (http://web.expasy.org/compute_pi/) (Sperandeo *et al.* 2005). Modeling of *RpP5cs* and *RpOat* genes was performed using SWISS-MODEL (https://swissmodel.expasy.org). N-glycosylation and O-glycosylation sites were evaluated using the following weblinks such as http://www.cbs.dtu.dk/services/NetOGlys and http://www.cbs.dtu.dk/services/NetNGlys, respectively. Protein transmembrane regions were analyzed using http://www.ch.embnet.org/software/TMPRED_form.htmL. Protein phosphorylation sites, N-acetaminophen (GlcNAc) protein modification sites, sumoylation modification sites, tyrosine sulfation sites of proteins and subcellular localization were examined with http://www.cbs.dtu.dk/services/NetPhos; http://www.cbs.dtu.dk/ services/YinOYang/; http://www.abgent.com/sumoplot; http://sulfosite.mbc.nctu.edu.tw/1.0/; and http://www.softberry.com/berry.phtmL?topic=protcompan&group=programs & subgroup=proloc. Hydrophobicity and hydrophilicity of proteins were predicted using http://web.expasy.org/protscale/.

The alignment of RpP5cs amino acid sequences and 39 homologues protein sequence in other species were performed using Clustal Omega, results were colour coded. Phylogenetic trees of the Clustal results were constructed using the neighbor-joining (NJ) method (MEGA6.0) (Tamura *et al.* 2013) using p-distance and complete deletion option parameters, and ascertained using bootstrapping methods with 1000 replicates. Multiple sequence alignment of amino acid sequences from *RpOat* and 11 other homologues genes were performed using the Clustal Omega, and the methods of phylogenetic trees were constructed as for the *RpP5cs* genes.

Total RNA was extracted from the samples with Trizol reagent (TransGen Biotech) and quantified with Nanophotometer^{TM} Pearl (Implen GmbH, Munich, Germany). RNA with OD260/280 ≥ 1.8 and the concentration ≥ 100 ng/µl was selected for the following experiment. The cDNA strand was synthesized using cDNA Synthesis SuperMix (TransGen Biotech) according to the manufactures' instructions. Gene-specific primers were designed (Table 2). One denaturation cycle was performed at 95°C 5 min, the qRT-PCR cycle was as follows for 40

amplification cycles: 95°C/30 sec; 40 cycles of 95°C/5 sec, and 60°C/30 sec. Each treatment was repeated at least three times independently, the *β-actin* gene was used as an internal control.

Table 2. Primer sequences for *RpP5cs* and *RpOat* genes for qRT-PCR.

Name	Sequence (5´-3´)
RpP5cs forward primer	TGGATACGAAGAATCACTTGTAGC
RpP5cs reverse primer	AGAAGCAATCTGAACTAAGGCATC
RpOat forward primer	TTATACCTGTCAGTGCTGTTCTTG
RpOat reverse primer	ATCCCTTAGTTCTTCTCCCATTTC
β-actin forward primer	GCTCATCCTGTCGGCAATAC
β-actin reverse primer	ACGAGACTACCTACAACTCCATC

All experiments were repeated at least three times. Statistical analyses and graph were performed using Microsoft Excel 2007 (Microsoft, USA). Each column means ± SE for three biological replicates (n > 3), Different letters indicated significant differences control and samples at time (P < 0.05).

Results and Discussion

Plant proline accumulation plays key roles in response to various stresses. The proline synthesis pathway can be divided into two species: glutamate pathway and ornithine pathway, P5CS and OAT are key enzymes in plants (Kavi Kishor *et al.* 2005). The *RpP5cs* and *RpOat* genes were identified using Blast 2.3 software by comparison to *Rumex patientia L.* transcriptome data (SRP11657). Sequences c61543_g1 and c75855_g1_i1 were selected as *P5cs* and *Oat* genes. The predicted conserved domains are shown in Fig 2, sequence c61543_g1 was predicted to be delta-1-pyrroline-5-carboxylate synthase and c75855_g1_i1 was predicted to be ornithine-delta-aminotransferase, the predicted results were similar to NCBI published gene results.

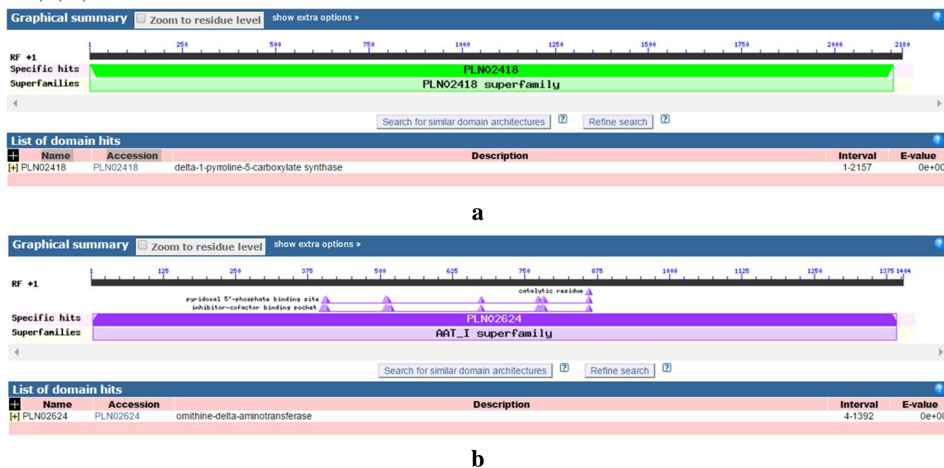


Fig. 2. The predicted conserved domains results of selected *RpP5cs* and *RpOat* genes. a. The predicted conserved domains results of selected *RpP5cs* genes; b. The predicted conserved domains results of selected *RpOat* genes.

The sequences of c61543_g1 and c75855_g1_i1 were used to design the gene specific primers, the homologous genes were amplified from *R. patientia* plants. Genes were predicted using BLASTN and conserved domain, then named *RpP5cs* and *RpOat* (Fig. 3). These results showed the open reading frame (ORF) of the *RpP5cs* gene contained 2178 nucleotides (bp) encoding 725 aa and the *RpOat* ORF contained 1401 nucleotides (bp) encoding 466 aa with sequencing. *P5cs* genes have been cloned from *Arabidopsis thaliana* (Thompson *et al.* 1997), *Brassica napus* (Kubala *et al.* 2015), *Phaseolus vulgaris* (Chen *et al.* 2013), *Medicago truncatula* (Armengaud *et al.* 2004) and other species (Armengaud *et al.* 2004, Su *et al.* 2011). *Oat* genes were sequenced in many species, e.g. *Vigna aconitifolia* (Delauney *et al.* 1993), other species (Hervieu *et al.* 1995), *Arabidopsis thaliana* (Nancy *et al.* 2002)

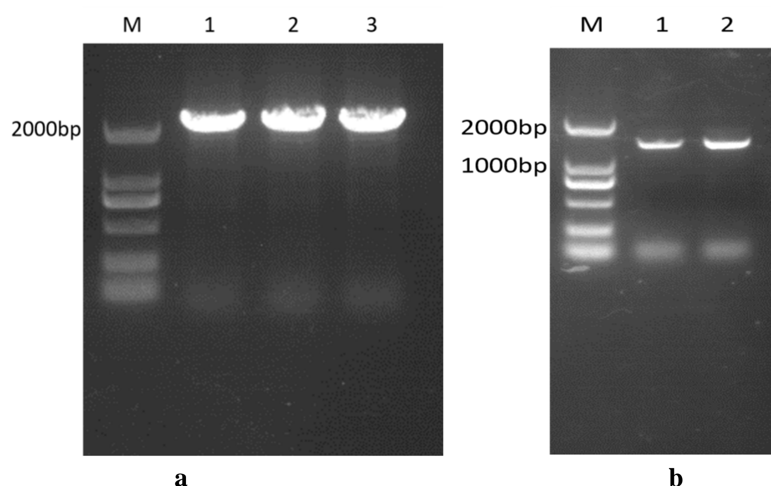
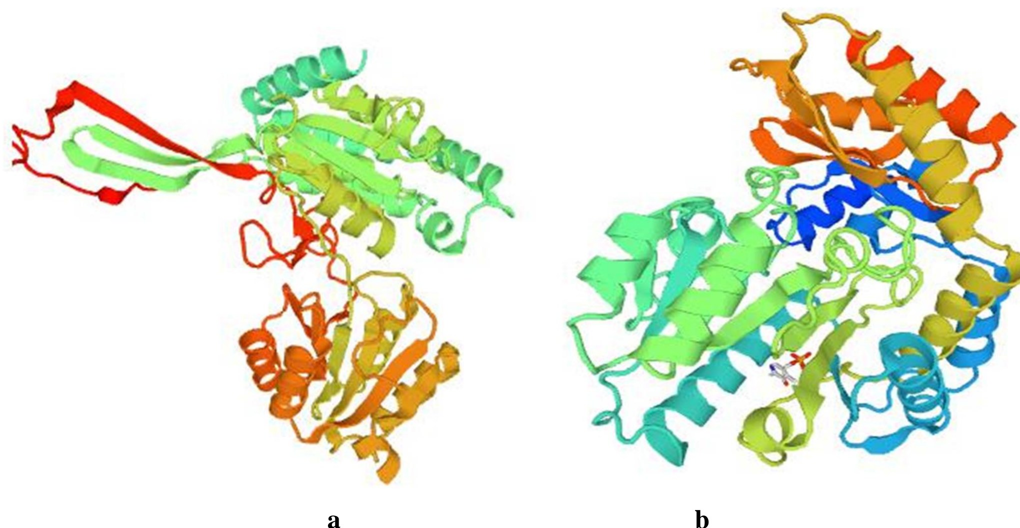


Fig. 3. Cloning of *RpP5cs* and *RpOat* genes. a. Cloning of *RpP5cs* gene; b. Cloning of *RpOat* gene.

The molecular characteristics of *RpP5cs* were predicted using the ProtParam tool and are shown in Table 3, the results showed 725 amino acids, molecular weight of 177916.65, pI = 4.95, and the formula was $C_{6578}H_{10980}N_{2178}O_{2776}S_{403}$. The RpP5CS protein was hydrophobic according to Protscale software. The RpP5CS protein had two kinds of transmembrane domains including 64 inside-outside transmembrane domains and 64 outside-inside transmembrane domains, which were located in the mitochondria. RpP5CS had 95 O-glycosylation sites and 23 N-glycosylation sites using NetNGlyc and NetOGlyc. It was also predicted to have 66 protein kinase phosphorylation sites including 39 serine phosphorylation sites, 18 threonine phosphorylation sites, and five tyrosine phosphorylation sites with NetPHos 3.1 Server, which also predicted seven O-GlcNAc glycosylated sites, seven small ubiquitin-like modifier sites, and three sulfation sites, when the three-dimensional model of RpP5CS protein was constructed using SWISS-MODEL software (Fig. 4a).

Table 3. The molecular characteristics of *RpP5cs* and *RpOat* genes.

Gene name	Amino acids	Molecular weight	pI	Formula
RpP5cs	725	177916.65	4.95	$C_{6578}H_{10980}N_{2178}O_{2776}S_{403}$
RpOat	466	51242.5	7.86	$C_{4217}H_{7035}N_{1401}O_{1772}S_{275}$



a b

Fig. 4. Tertiary structure prediction of proteins for *RpP5cs* and *RpOat* genes. a. Tertiary structure prediction of *RpP5cs* gene protein; b. Tertiary structure prediction of *RpOat* gene protein.

The molecular characteristics of *RpOat* were predicted using the ProtParam tool and are shown in Table 3, the results showed 466 amino acids, molecular weight of 51242.5, pI = 7.86, and the formula was $C_{4217}H_{7035}N_{1401}O_{1772}S_{275}$. The RpOAT protein was hydrophobic according to Protscale software. The RpOAT protein had two kinds of transmembrane domains including 42 inside-outside transmembrane domains and 39 outside-inside transmembrane domains, which were located in the mitochondria or cytoplasm. RpOAT had 95 O-glycosylation sites and 12 N-glycosylation sites using NetNGlyc and NetOGlyc. It was also predicted to have 30 protein kinase phosphorylation sites including 19 serine phosphorylation sites, seven threonine phosphorylation sites, and four tyrosine phosphorylation sites with NetPHos 3.1 Server, which predicted seven O-GlcNAc glycosylated sites, nine small ubiquitin-like modifier sites and one sulfation site (Fig S11b), when the three-dimensional model of RpP5CS protein was constructed using SWISS-MODEL software (Fig. 4b).

The best sequences from BLAST search were used to generate two phylogenetic trees (Fig. 5). The results showed the P5cs protein of *R. patientia*, *Salicornia bigelovii* (sea tent) and *Mesembryanthemum crystallinum* (published in Genbank) clustured on a single branch of the tree, and was distinct from other plants; the *R. patientia* and *Solanum lycopersicum* (tomato) gene were clustered on the same evolutionary branch, which was distinct from 11 other species.

RpP5CS protein domain analysis showed the presence of two conserved domains including AAK superfamily accession number CD04256 and ALDH_F18-19_Pro-GPR accession number CD07079. RpOAT protein domain analysis showed one conserved domain in AAT_I superfamily, with Pfam accession number PLN02624 as reported earlier (Ramadan and Hassanein 2014, Marchler-Bauer *et al.* 2017). RpP5CS was located in mitochondria and RpOAT was located in mitochondria or cytoplasm (Nancy *et al.* 2002, Ramadan and Hassanein 2014). Based on the above analyses, characteristic analysis of *RpP5cs* and *RpOat* genes were predicted and phylogenetic analyses were constructed to provide a theoretical basis for future studies.

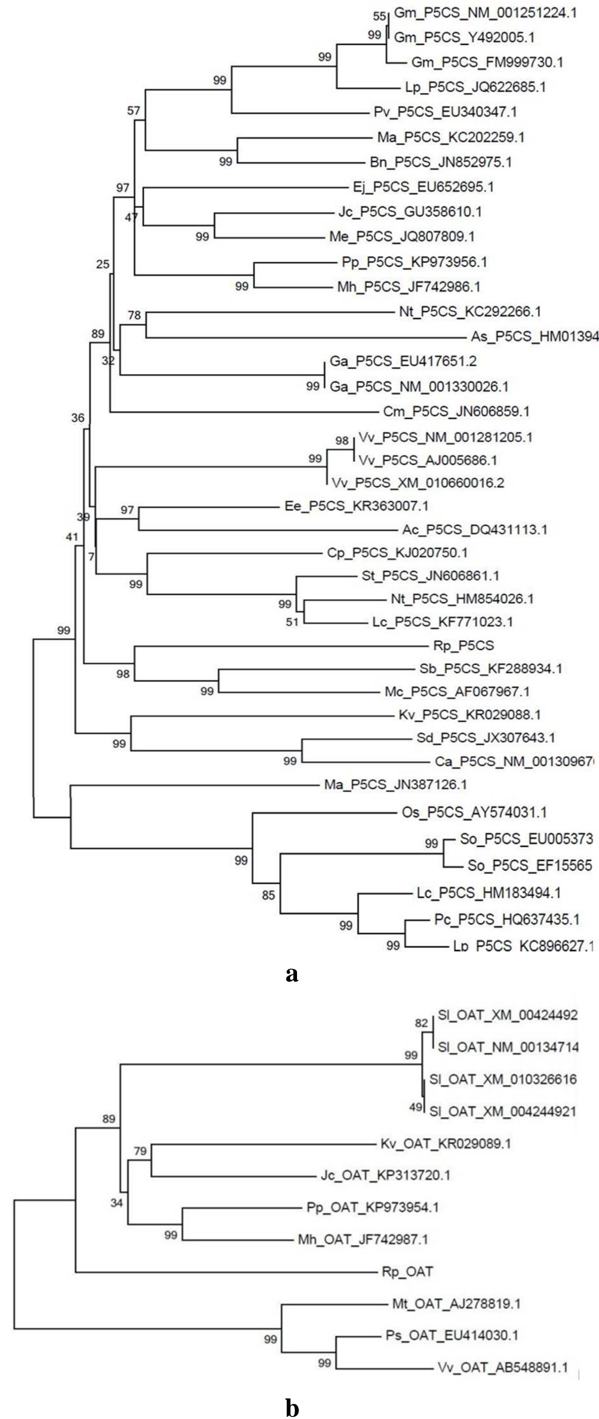


Fig. 5. Phylogenetic trees constructed with MEGA6 based on the amino acids of the *RpP5cs* and *RpOat* genes from different plant species. a. *RpP5cs* gene; b. *RpOat* gene.

The expression of *RpP5cs* and *RpOat* genes in Russian Щавель Чемпион (L) and Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex (as control) were challenged with salinity and alkalinity stress, the results showed the expression of *RpP5cs* genes were higher in Russian Щавель Чемпион than Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex, and the expression of *RpP5cs* gene was highest in Russian Щавель Чемпион at 6 h; the expression of *RpP5cs* was down-regulated in Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex (Fig. 6a). The expression of *RpOat* gene first increased and then decreased in Russian Щавель Чемпион (L.), and the expression of *RpOat* was highest in Russian Щавель Чемпион at 12 hrs, the expression of *RpOat* was higher in Russian Щавель Чемпион than Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex. The results suggest Russian Щавель Чемпион could be more resistant to salt and alkalinity compared with Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex (Fig. 6b). The results showed that Russian Щавель Чемпион (L) is a resistant species to the salt and alkalinity.

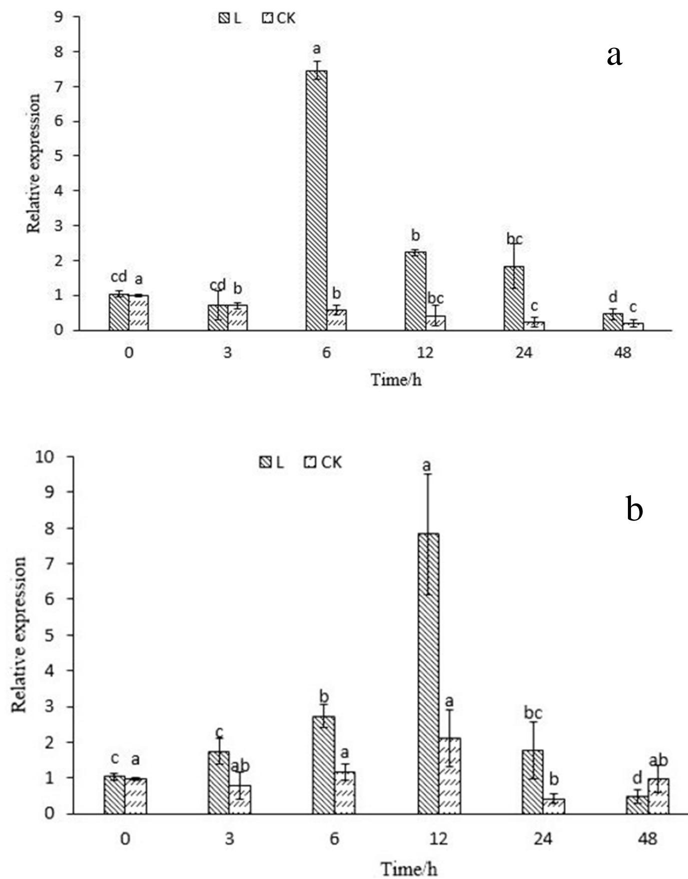


Fig 6 Relative expression of *RpP5cs* and *RpOat* genes under complex saline-alkali conditions. a. Relative expression of *RpP5cs* gene under complex saline-alkali conditions; b. Relative expression of *RpOat* gene under complex saline-alkali conditions. L show Russian Щавель Чемпион; CK represented Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex. 0h as control. Each column means ± SE for three biological replicates ($n > 3$). Different letters indicated significant differences control and samples at time ($p < 0.05$).

Expression of *RpP5cs* and *RpOat* genes could be changed by various abiotic stresses. The expression of *NtP5cs* gene was induced by salt, dehydration, and temperature stress (Zheng *et al.* 2014). Over-expression of *PvP5cs1* and *PvP5cs2*in *Arabidopsis* increased proline production and salt tolerance (Chen *et al.* 2013). OAT activity was increased with salt treatment, the expression of *AtOat* gene increased after exposure to 200 mM NaCl (Canas *et al.* 2008). Radish (*Raphanus sativus*) seedlings using the OAT inhibitor demonstrated the contribution of the ornithine pathway to proline synthesis under NaCl treatment (Hervieu *et al.* 1995). In previous studies, the *P5cs* and *Oat* genes of plants decreased under salinity and alkalinity treatment. In the present study, the expression of *RpP5cs* and *RpOat* genes were higher in Russian Щавель Чемпион than Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex under salinity and alkalinity treatment over 24 hrs. The results suggest Russian Щавель Чемпион could be more resistant to salt and alkalinity compared with Chinese *Rumex patientia* × *R. tianschanicus* cv. Rumex, as the expression of *P5cs* and *Oat* genes was measured to determine the capability to respond to various abiotic stresses.

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