Genomics and Genetics 2022;15(2): 55–63

Potential Role of *Peptide Transporter2* Gene in Salt Stress Response

Achmad Syarifudin[1], Supachitra Chadchawan[1*]

[1]*Center of Excellence in Environment and Plant Physiology, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*

[*]*Correspondence: Supachitra.c@chula.ac.th; s_chadchawan@hotmail.com*

ABSTRACT

The Thai rice cultivar 'Luang Pratahn' is a promising candidate for a salt-tolerant donor plant. Previously, several key genes responsible for salt tolerance were identified using time-course transcriptomic data and weighted co-expression networks. The rice peptide transporter2 (*OsPTR2*) gene is one of the key genes with no prior information related to the salt tolerance mechanism in rice. Salt stress could induce *OsPTR2* gene expression in salt tolerant cultivars, 'Pokkali' and 'Luang Pratahn', while it could not be induced in IR29, the salt susceptible line, suggesting the possibility of *OsPTR2* involvement in salt tolerance. Therefore, this research aims to investigate the role of PTR2 gene in salt stress response by using the *OsPTR2* orthologous gene in Arabidopsis, *AtPTR2*. Based on bioinformatic analysis, *OsPTR2* and *AtPTR2* are transmembrane proteins and share 5 similar domains. Therefore, the homozygous line of *Atptr2* knocked-out mutant line was used to investigate the involvement of *Atptr2* gene in salt stress response. After 7 days under salt stress condition, *Atptr2* mutant showed significantly shorter root length and lower dry weight than wild type (WT), without significant difference in photosynthetic pigment contents between the mutant and WT. These results suggested that *Atptr2* had a potential role in root development under salt stress condition. The revertant of *Atptr2* mutant by *OsPTR2* expression will be performed for further characterization.

Keywords: 'Luang Pratahn'; mutant; salt stress; peptide transporter; *PTR*

INTRODUCTION

Rice is one of the most important foods in the world, especially in ASEAN countries (Jackson, 1997). The high salinity of the soil leads to a gradual decline in the area under rice cultivation (Katawatin & Sukchan, 2012). The causes of soil salinity include seawater contamination and the misuse of fertilizers during cultivation (Park *et al*., 2022).

The researcher aims to solve the problem and increase the salt tolerance of rice by using several approaches. A molecular breeding program (MBP) is one of the best approaches. Compared with the conventional method, MBP has a fast and high rate of success and can stabilize important traits over generations. QTL mapping is one example of a very successful technology within the MBP (Amoah *et al*., 2020). Although QTL mapping is a great approach, the variety that can serve as the donor trait is crucial for the new tolerance cultivars (Habila *et al*., 2022; Liu *et al*., 2022). Habila et al (2022) investigated 8 Thai landrace rice cultivars in comparison with the salt tolerant standard variety 'Pokkali' and salt susceptible line, IR29 and found that 'Jao Khao', 'Lai Mahk' and 'Luang Pratahn' were tolerated to salt stress in comparable to 'Pokkali' at seedling stage. Therefore, these cultivars can be used as a donor variety in plant breeding program.

'Luang Pratahn' is an Indica rice cultivar originally grown in Thailand. This variety has great potential to serve as a donor for salt tolerance new cultivar development using molecular breeding programs. The *OsPTR2* gene is one of the genes identified based on the gene co-expression network in 'Luang Pratahn' Thai rice cultivar (Sonsungsan *et al*., 2021). The *OsPTR2* gene, or *protein transporter2,* is a gene related to the transport of nitrate and small peptide (Yang *et al*., 2020).

Characterization of *PTR* genes was reported in the rice (Zhao *et al*., 2010). As one of the *PTR* gene family members, the *PTR2* gene is involved in the rice nitrogen uptake and utilization (Yang *et al*., 2020). Mutation of the *OsNPF2.2* caused abnormal vascular structure and slowed plant development (Li *et al*., 2015). Another work has been reported on the characterization of the PTR Arabidopsis mutants. In Arabidopsis, loss of function PTR2 mutant shows lower water content and higher free ABA content. Thus, the overexpression and complementation of PTR2 show positive feedback by increasing the water uptake during early seed germination and lowering the

Received 17 November 2022, Revised 1 December 2022, Accepted 13 December 2022

free ABA content (Choi *et al*., 2020; Yang *et al*., 2020). So far there have been no reports related to the involvement of the *PTR2* gene during salt stress responses.

In this study, we confirmed the importance of this gene by examining the gene expression patterns in rice as well as the orthologous phenotype with a knock-out version in the Arabidopsis mutant. These results are important to becoming a criterion for gene selection and can be used as a revertant in the future.

MATERIALS AND METHODS

Phylogenetics analysis of *PTR2*- related gene family from rice and Arabidopsis

The phylogenetic tree was generated using iTOL software https://itol.embl.de. using sequence retrieved from the MSU rice genome database (http://rice.uga.edu) for rice and TAIR (https://www.arabidopsis.org) for the Arabidopsis. Rice PTR genes were retrieved by using BLASTP searching tools of the peptide transporter amino acid conserved sequence, following that manual checking was conducted by searching for the same keyword in the putative function tools, and the lists of the gene were downloaded by the genome annotation batch downloader tool. Arabidopsis *PTR* genes were retrieved by searching the peptide transporter keyword on the gene option searching tool, and lists of the gene were downloaded by the bulk sequence downloader tool.

Sequence conserved domain of *OsPTR2* and *AtPTR2* gene

The sequence conserved domains were created by using NCBI tools https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. By using the same sequences gathered from BLASTP we retreived the sequence conserved domain in both organisms.

Prediction of 3D structural model *OsPTR2* & *AtPTR2*

The protein sequences of *OsPTR2* and *AtPTR2* were retrieved from the rice genome database (*http://rice.uga.edu*) and TAIR (*https://www.arabidopsis.org*). The sequences were used to generate a putative 3D model protein using AI tools (*AlphaFold.jpynb*). The refinement of the predicted 3D model was performed using modrefiner (Xu & Zhang, 2011). The refined 3D model was then analyzed using UCFS ChimeraX (*https://www.cgl.ucsf.edu/chimera/*). The gene structure using CDS and genome sequences of both genes, and the analysis using GSDS 2.0 (Hu *et al.*, 2015). The motif analysis uses MEME Suite 5.4.1 (Bailey *et al.*, 2015) and the retrieved motif sequences were mentioned in the putative protein 3D model.

Gene expression analysis of *OsPTR2*

Plant materials and growth condition

Hundreds of rice seeds of 'Pokkali' (a salt-tolerant cultivar), 'Luang Pratahn' (a salt-tolerant candidate cultivar), and IR29 (a salt-susceptible cultivar) were provided from the CEEPP Laboratory, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. Seeds were sterilized and germinated on the plates. Seven-day-old seedlings were planted with the sandy-like soil obtained from Pimai District, Nakhon Ratchasima province, Thailand, in 3-inch pots. Three biological replications were used in the experiment with a randomized complete block design (RCBD). The twenty-day-old rice seedlings are further treated with 115 mM NaCl solution to generate the salt stress condition with the soil EC of 9–10 dSm^{-2} (Kojonna *et al*., 2022) and compared with the seedlings grown in the normal condition, which were used as controls. All leave tissues from each plant were collected at 0, 3, 6, 12, 24, and 48 hours after the treatments and stored at -80 °C for RNA extraction.

Total RNA extraction and cDNA synthesis

The leaf samples are ground with a mortar and pestle. The extraction steps follow the protocol of GENEzol GZR100 (Geneaid Biotech., Taiwan). The RNA samples are treated with DNAse I (Thermo Scientific, USA) to remove DNA contamination. The DNAse I treated RNA samples were used as template for the cDNA synthesis using iScript (New England Biolabs Inc., USA). Then, the cDNA samples were used for the gene expression analysis using the CFX Connect Real-Time PCR Detection System (Bio-Rad Inc., USA).

Gene expression analysis (qRT-PCR)

The *OsPTR2* (*LOC_Os10g22560*) cDNA sequence was retrieved from the rice gene annotation database and the IDTPrimer tool was used for primer design (https://www.idtdna.com/pages/tools/primerquest). cDNA samples were used to determine the level of *OsPTR2* gene expression using *OsEF-1α* gene expression level as the internal control (Chutimanukul *et al.*, 2018). *OsPTR2* gene expression was detected by primers and condition indicated in Table 1. Three technical replications were performed for each sample. Luna Universal qPCR Master Mix (New England Biolabs Inc., USA) was used for gene expression detection and relative gene expression was analyzed according to the Pfaffl formular (Pfaffl, 2001). Analysis of variance (ANOVA) of the gene expression levels among cultivars was performed and mean values of gene expression levels were compared using Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

Selection of *Atptr2* homozygous line

Plant growing condition and DNA extraction

Atptr2 mutant (SAIL_65_B10) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). They were germinated and cultured for seven days on MS medium (Murashige & Skoog, 1962) before transplanted into 3-inch pots containing a medium of perlite, vermiculite, and peat moss at 1: 1: 3 ratio. The experiment was held in the growth room with 60% relative humidity, 35 $\mu mol.m^{-2}.s^{-1}$ light intensity and 16:8 light/dark cycle at 23 °C. Three leaves of ten-day-old seedlings were collected for genomic DNA (gDNA) extraction according to Edward et al (1991) with some modification. Briefly, the frozen leaf samples were ground into powder with liquid nitrogen and then, 500 μL of extraction buffer (200mM Tris-Cl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The homogenized sample was immersed in water bath at 60 °C before centrifugation at maximum speed to precipitate plant tissue debris. DNA was obtained by adding an equal volume of absolute isopropanol to the supernatant. Then, the DNA pellet was washed with 70% ethanol and air dried at room temperature before dissolved in 20 μL of dH_2O and stored at -20 °C.

Atptr2 homozygosity testing

Primers for homozygosity testing, including Left Primer (LP), Right Primer (RP), and Left T-DNA Border Primer (LB) indicated in Table 1, are obtained from SIGnAL (*http://signal.salk.edu/tdnaprimers.2.html*). The gDNA was used as a template for amplification with the LP-RP primers and LB-RP primers to determine the homozygosity of the mutant lines. The polymerase chain reaction conditions are indicated in Table 1. Then, the seeds of the homozygous plants are collected for further analysis.

Phenotyping of salt response of *Atptr2*

Both wild type (WT) and selected *Atptr2* mutant Arabidopsis seeds were germinated on MS medium as indicated above for 7 days. Then, they were transferred to the freshly prepared MS medium or MS medium supplemented with 150 mM NaCl for salt stress treatment, which was the NaCl concentration that could clearly reveal the different responses between WT and the mutant line. The comparison of fresh weight, dry weight, photosynthetic pigment contents and root length were performed with a randomized complete block design (RCBD) with three biological replications. Analysis of variance of each phenotype was performed and mean comparison was done with Duncan's multiple range test (DMRT) at $p < 0.05$.

Fresh weight and dry weight were determined with 15 seedlings for each treatment and photosynthetic pigment contents, chlorophyll *a*, chlorophyll *b* and carotenoids, were determined according to Wellburn, 1994 (Wellburn, 1994). Briefly, the pigments were extracted with 80 % acetone overnight, then the pigment contents were determined according to the following formula:

$$\text{Chlorophyll } a \text{ (Chl } a\text{) content} = 12.25A_{663.2} - 2.79A_{646.8}$$
$$\text{Chlorophyll } b \text{ (Chl } b\text{) content} = 21.5A_{646.8} - 5.1A_{663.2}$$
$$\text{Total carotenoids} = (1000A_{470} - 1.82\text{Chl } a - 85.02\text{Chl } b)/198$$

Table 1 Primers and polymerase chain reaction conditions used in this research

Target of Detection	Primers	PCR condition	Size of amplified fragment
OsPTR2 (*LOC_Os10g22560*) gene expression	Forward primer: 5'GCCTTCTTGGCTGACACATA3' Reverse primer: 5'AAAGGTAGAGCCCTAGGTAGAC3'	Initial denaturation: 95ºC, 3 min. Denaturation: 95ºC, 10 sec. Annealing : 60ºC, 30 sec. Extension : 72ºC, 5 sec. Cycles : 40	172 bp
OsEF-1α gene expression	Forward primer: ATGGTTGTGGAGACCTTC Reverse primer: TCACCTTGGCACCGGTTG	Initial denaturation: 95ºC, 3 min. Denaturation: 95ºC, 10 sec. Annealing : 60ºC, 30 sec. Extension : 72ºC, 5 sec. Cycles : 40	127 bp
Atptr2 mutant homozygosity testing	LP: 5'TCGAAATCCGGAGATTATTCC3' RP: 5'TCTTCACATTTCCATGGAAGG3' LB: 5'GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC3'	Initial denaturation: 95ºC, 3 min. Denaturation: 95ºC, 30 sec. Annealing: 53ºC, 30 sec. (LP-RP) 52ºC, 30 sec. (LB-RP) Extension : 72ºC, 55 sec. Cycles : 35 Final extension: 72ºC, 5 min.	1263 bp ~900 bp

RESULTS

PTR gene family in rice and Arabidopsis

Peptide transporter 2 (*PTR2*) belongs to the nitrate and peptide transporter family (NPF), which is one of the largest transporter family in the plant kingdom (Kanstrup and Nour-Eldin, 2022). There are 70 and 52 NPF genes in rice and Arabidopsis, respectively (Longo *et al*., 2018). However, the information in the current databases is different from the previously reported. *AtPTR2* was identified *via* complementation test of yeast peptide transport mutant, which allowed the yeast growth on dipeptide and tripeptide supplements (Steiner et al., 1994). In rice genome database (Ouyang *et al*., 2007), 73 loci of peptide transport-related genes are annotated. Fifty of them were annotated as *PTR2* gene (Supplementary Table 1). When the amino acid sequence of *LOC_Os10g22560* (*OsPTR2*) was used to blast against *Arabidopsis thaliana* genome 20 homologous loci were reported and *AT2G2040* (*AtPTR2*) showed the highest similarity of 66%. Among 20 homologous loci in Arabidopsis, one of them does not have the polypeptide sequence, suggesting that it is the *PTR* pseudo-gene. Therefore, a total of 73 rice peptide transporter proteins and 19 Arabidopsis peptide transporter proteins were subjected to phylogenetic analysis.

Based on phylogenetic analysis, it reveals 5 clades of the peptide transporter proteins (Figure 1). At least 1 *AtPTR* was a member in each clade, except Clade II, which contains no *AtPTR*, suggesting that *OsPTR* genes in clade II evolved after rice genome evolution from the dicot progenitor. Group I (Clade I) consists of 12 genes with 1 gene belonging to Arabidopsis. Group II (Clade II) consists of 12 rice genes only, while 5 rice genes and a single gene from Arabidopsis are in Group III (Clade III). Group IV (Clade IV) is the second largest group, containing 15 *PTR* rice genes and 12 *PTR* gene from Arabidopsis. Group V is the largest group with 34 genes, 29 of them belong to rice and 5 genes are Arabidopsis genes. *OsPTR2* (*LOC_Os10g22560*) is located in clade V, where the closest relative from Arabidopsis is *AT2G02040* or *AtPTR2*. Therefore, the mutant line with the insertion at *AT2G02040*, SAIL_65_B10 (*Atptr2*), was selected to determine the involvement of the *AtPTR2* gene in salt tolerance in order to infer the role of *OsPTR2*.

OsPTR2 (*LOC_Os10g22560*) and *AtPTR2* (*AT2G02040*) contain the conserved domains, spanning from 115 bp to 580 bp in *AtPTR2* and 115 bp to 525 bp in *OsPTR2*. *AtPTR2* contains 4 exons and 3 introns, while *OsPTR2* has 5 exons and 4 introns. *OsPTR2* also has a longer 5'UTR than *AtPTR2* (Figure 2 A). Both PTR2 proteins have 5 consensus motifs as shown with different colors, red, cyan, green, purple and orange, in Figure 2 B and C. The repeated folding of α-helix structure of both proteins suggests the transmembrane location.

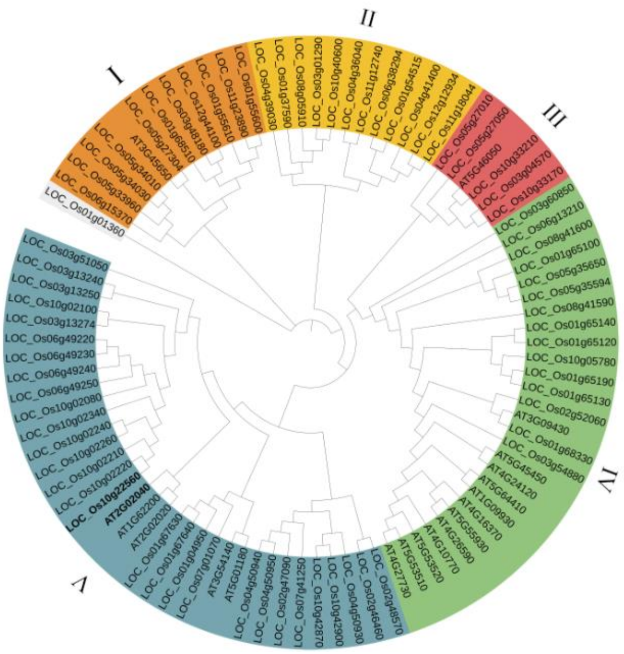

Figure 1 Phylogenetic analysis of *PTR2*- related gene family from rice and Arabidopsis.

Expression analysis of *OsPTR2* gene

In order to determine the salt-induced gene expression in rice, three cultivars, 'Pokkali', the salt-tolerant standard cultivar, 'Luang Pratahn', a salt-tolerant Thai rice cultivar, and IR29, the salt susceptible line, were investigated at seedling stage. Salt stress-induced gene expression of *OsPTR2* could not be detected in 'Pokkali' rice during salt stress treatment (Figure 3 A), while salt stress could significantly induce *OsPTR2* after 6 hours of the treatment. Then, it was suppressed after 24 hours of the treatment (Figure 3 B). On the other hand, no significant salt-stress induction of *OsPTR2* was detected in IR29 rice (Figure 3 C).

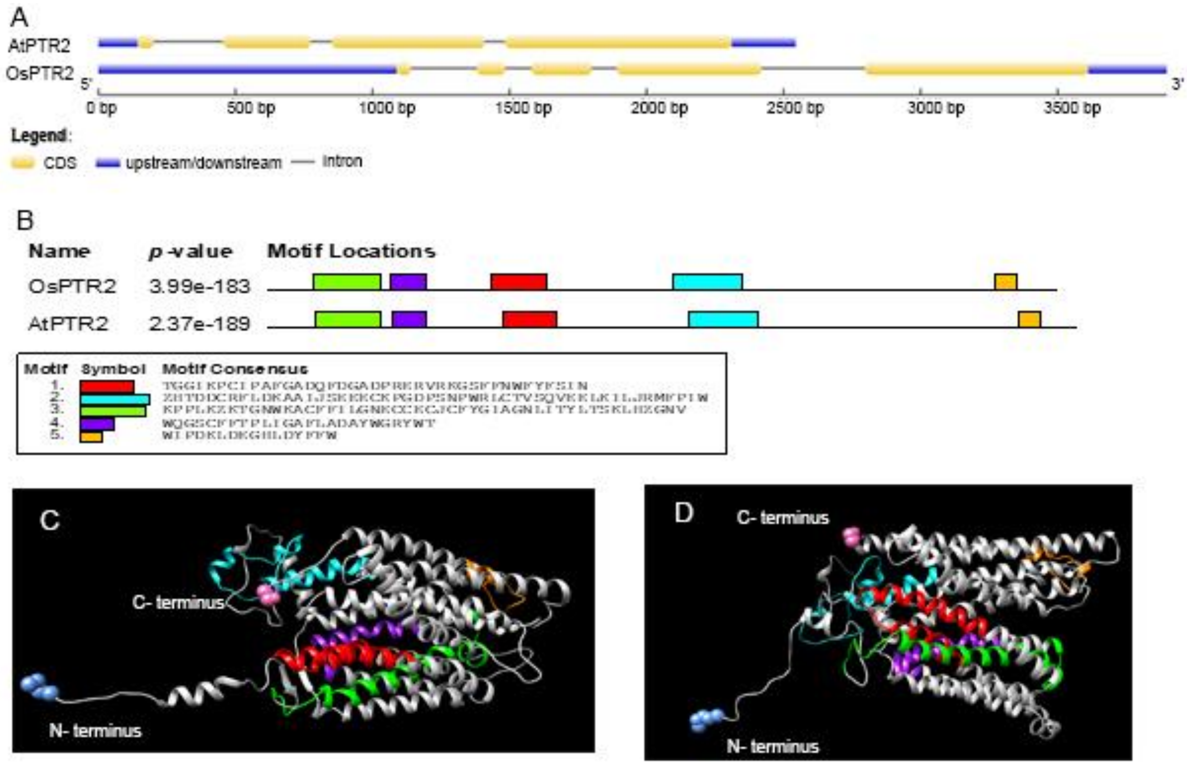

Figure 2 The gene models of *AtPTR2* and *OsPTR2* (A) with the demonstration of the consensus motifs in both proteins (B) are demonstrated. The 3-D models of *AtPTR2* (C) and *OsPTR2*(D) predicted by *AlphaFold2.ipynb* are shown.

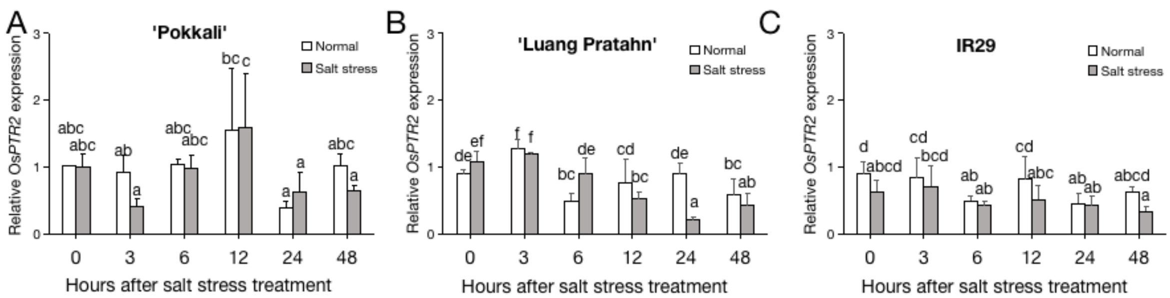

Figure 3 *OsPTR2* gene expression in 'Pokkali' (A), 'Luang Pratahn' (B) and IR29 (C) rice seedling, when grown in normal and salt stress conditions for 0, 3, 6, 12, 24, and 48 hours. The *OsPTR2* gene expression was calculated in relative to *OsEF1α* gene expression.

Salt stress response phenotypes of *Atptr2* Arabidopsis mutant

Atptr2 mutant line, SAIL_65_B10, provided by TAIR, was analyzed for salt-responsive traits in comparison with wild type (WT). SAIL_65_B10 seed stock contains a mixture of homozygous and heterozygous T-DNA insertion. In order to select the homozygous insertion lines, amplification of the genomic DNA with two pairs of primers, LP-RP and LB-RP primers, were used. The positive 1.2 kb fragment amplified with LP-RP primer indicates the *AtPTR2* WT genotype, while 0.9 kb fragment amplified with LB-RP primers indicates the existence of T-DNA insertion at *AtPTR2* (Figure 4 A).

Based on the amplification with LP-RP and LB-RP primers, 2 out of 10 lines, L3 and L9, were detected as the homozygous T-DNA insertion lines at *AtPTR2* gene as no amplification could be detected when they were amplified with LP-RP primers (Figure 4 B) and LB-RP primers could be used to amplify the 0.9 kb fragments (Figure C). In some cases, both primer pairs could not be used to amplify any specific bands for example L1, while the positive bands were detected by both primer pairs in some lines, L4, L5, L6, which revealed the heterozygous genotype. Moreover, we found out that WT seeds were mixed in this seed collection as shown in L2, L7, L8 and L10 (Figure 4 B and C). The homozygous insertion lines then were grown to collect the seeds for the salt stress response phenotyping. In this research, L3 was selected for phenotyping responses to the salt stress.

Phenotypes of *Atptr2* mutant line under salt stress condition

Arabidopsis WT and *Atptr2* mutant seeds were germinated on the MS medium plates for 7 days. Then, the seedlings were transferred to grow on the MS medium or MS medium supplemented with 150 mM NaCl. After 7 days of the treatment, the seedlings' fresh weight, dry weight, photosynthetic pigment contents and root length were determined.

Salt stress inhibited both WT and *Atptr2* mutant seedling growth, but the stronger effect was detected in the mutant lines (Figure 5 A and B). Dry weight of the mutant line was significantly lower than WT seedlings (Figure 5 B). Salt stress decreased the photosynthetic pigments, chlorophyll *a* (Figure 5 C), chlorophyll *b* (Figure 5 D) and carotenoids (Figure 5 E) in both WT and *Atptr2* mutant seedlings. Interestingly, salt stress showed more effect in root growth inhibition in the *Atptr2* mutant (Figure 5 F, G and H).

DISCUSSION

Based on this PTR phylogenetic analysis, 5 clades (groups) of the PTR proteins are revealed. The number of PTR clades is consistent with the report by Yang *et al*. (2020). However, Yang *et al*. (2020) retrieved 96 PTR proteins from 'Nipponbare' (*Oryza sativa* L. subsp. *Japonica*), 85 PTR proteins from R498 (*Oryza sativa* L. subsp. *Indica*), and 78 proteins from *Oryza glaberrima* for their analysis. Some of them (29 loci) were excluded from our study, because they are not annotated as PTR or PTR-like genes by Ouyang *et al*. (2007). However, 6 PTR proteins included in this study were excluded from Yang *et al*. (2020). *OsPTR2* (*LOC_Os10g22560*), shown in Group V in our study, was located in sub-group Vd (Yang et al., 2020). The PTR proteins in sub-group Vc and Vd analyzed by Yang *et al*., (2020) belong to Group V in this study.

OsPTR2 (*LOC_Os10g22560*) and *AtPTR2* (*AT2G02040*) are symporter proteins and also known as *Peptide transporter2* (*PTR2*). It belongs to the major facilitator superfamily (MFS) (Yang *et al*. 2020), which facilitates the transport of small solutes across the membrane in response to chemiosmotic gradients (Pao *et al*., 1998). *OsPTR2* encodes a proton-dependent symporter protein found in the cell membrane (Ouyang *et al*., 2010). Another *OsPTR2* gene (*LOC_Os01g04950*), a member in Group V and sub-group Vb (Yang et al., 2020), was shown to be up-regulated by salt stress in salt-tolerant rice, suggesting a role in the salt tolerance (Amanat *et al*., 2022). This supports the potential of *OsPTR2* (*LOC_Os10g22560*) to participate in salt tolerance.

The bioinformatics studies started with the analysis of the gene structure, we discovered different structures in rice and Arabidopsis, implying that duplication and differentiation might happen during the evolution and changed the length of the sequences and the structure (Pusadee *et al*., 2009). Based on 3D model predictions, OsPTR2 and AtPTR2 share five conserved motifs. These motifs are related to the POT family which is another name for the PTR family. This family functions not only related to the nitrate transporter, but also the transportation of di/tripeptides in the plant (Chiang *et al*., 2004; Prabhala *et al*., 2021).

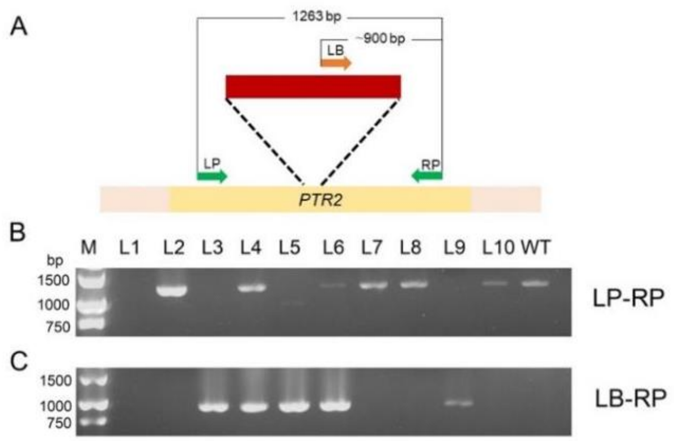

Figure 4 The above diagram shows the primer pairs and sizes of the amplified fragments using for the detection of the T-DNA insertion in *Atptr2* mutants (A). Ten lines, L1-L10 genomic DNA was detected with LP-RP (B) and LB-RP primers (C).

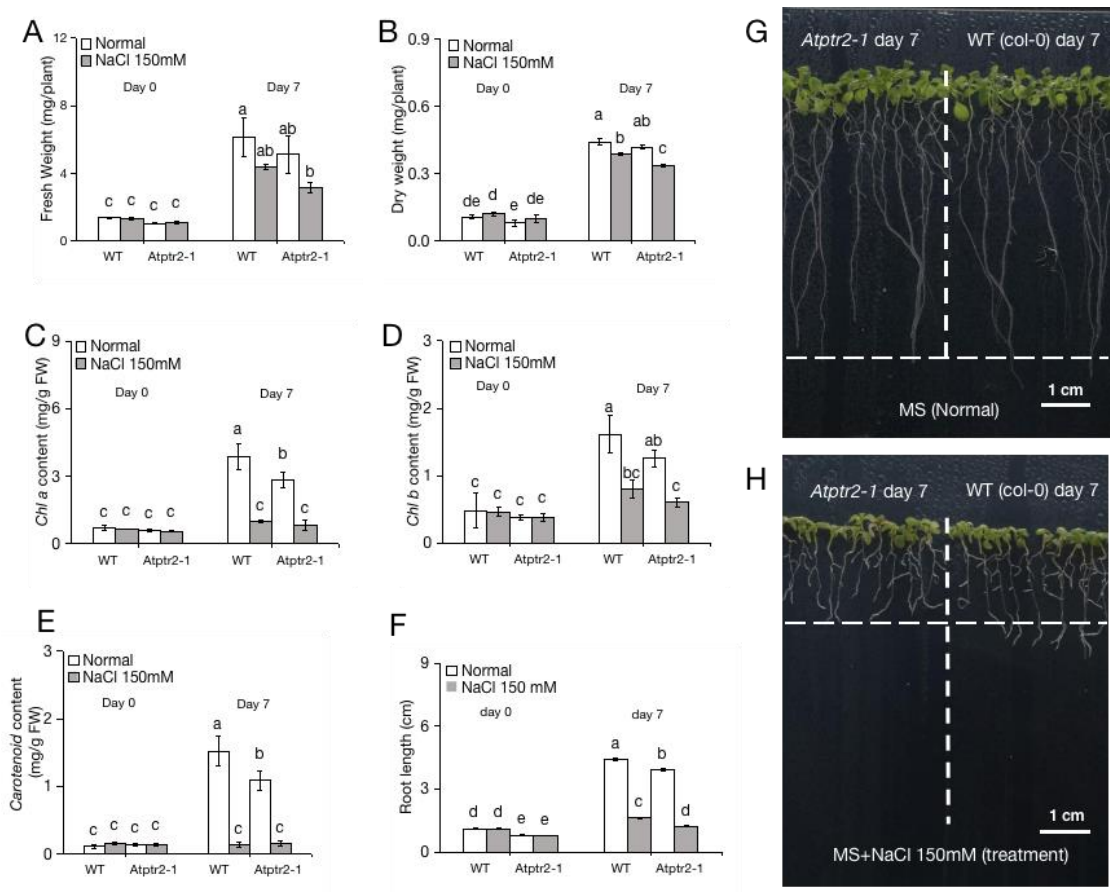

Figure 5 Seven-day old *Atptr2* and WT seedlings were grown in MS medium or MS medium supplemented with 150 mM NaCl for 7 days. Then, fresh weight (A), dry weight (B), photosynthetic pigments, chlorophyll *a* (C), chlorophyll *b* (D), and carotenoids (E) were determined, including root length (F). The phenotypes of the seedlings in normal (G) and salt stress (H) conditions are demonstrated. The horizontal dash lines in G and H represent the average root length of *Atprt2* mutant. The different letters above the bar indicate the significant difference between the means by DMRT analysis at $p<0.05$.

At least two peptides, systemin (Cirillo *et al*., 2022) and *AtPep3* (Nakaminami *et al*., 2018) were demonstrated to have a role in salt tolerance in plants. Systemin is synthesized as prosystemin and it was shown to play a role in both biotic and abiotic stress. It was suggested that it represented the cross-talk between biotic and abiotic stress response in tomato (Cirillo *et al*., 2022). C-terminal peptide fragment (*AtPep3*) encoded from *AtPROPEP3* could inhibit the salt-induced bleaching of chlorophyll in seedlings and the knock-down *AtPROPEP3* plant exhibited a hypersensitive response to salinity stress, which could be complemented with application of *AtPep3* peptide (Nakaminami *et al*., 2018). These data suggest that peptide transporter may have a role in the transport of the specific peptide molecules to adapt themselves to salt stress.

The significant salt-stress induction of *OsPTR2* was found only in 'Luang Pratahn' rice, suggesting that the action of *OsPTR2* was quite specific to 'Luang Pratahn' rice and may contribute to salt tolerance in this cultivar (Figure 3). Based on the motif similarity between OsPTR2 (LOC_Os10g22560) and AtPTR2 (AT2G02040) (Figure 2), the *Atptr2* mutant line was selected to investigate the salt stress response. The more salt stress susceptibility in *Atptr2* mutant determined by fresh weight, dry weight and root length supports the role of the *Atptr2* gene in salt tolerance. This is the first evidence for *PTR2* gene involvement in salt stress response. Therefore, *OsPTR2* is proposed to be involved in salt tolerance. How this gene facilitates salt tolerance should be further investigated in the future.

ACKNOWLEDGEMENTS

This research is supported by Thailand Science Research and Innovation Fund Chulalongkorn University (CU_FRB65_food (5) 113_23_43). AS is supported by Asean and Non-Asean Scholarship 2020.

REFERENCES

Amanat MA, Naeem MK, Algwaiz HIM, Uzair M, Attia KA, AlKathani MDF, Zaid IU, Zafar SA, Inam S, Fiaz S, *et al*. Evaluation of Green Super Rice Lines for Agronomic and Physiological Traits under Salinity Stress. Plants. 2022;11(11):1461.

Amoah NKA, Akromah R, Kena AW, Manneh B, Dieng I, Bimpong IK. Mapping QTLs for tolerance to salt stress at the early seedling stage in rice (*Oryza sativa* L.) using a newly identified donor 'Madina Koyo.'. Euphytica. 2020;216(10):156.

Bailey TL, Johnson J, Grant CE, Noble WS. The MEME Suite. Nucleic Acids Res. 2015; 43(1):W39-W49.

Chiang CS, Stacey G, Tsay YF. Mechanisms and functional properties of two peptide transporters, *AtPTR2* and *fPTR2*. J Biol Chem. 2004;279(29):30150–30157.

Choi MG, Kim EJ, Song JY, Choi SB, Cho SW, Park CS, Kang CS, Park YI. *Peptide transporter2* (*PTR2*) enhances water uptake during early seed germination in *Arabidopsis thaliana*. Plant Mol Biol. 2020;102(6):615–624.

Chutimanukul P, Kositsup B, Plaimas K, Buaboocha T, Siangliw M, Toojinda T, Comai L, Chadchawan S. Photosynthetic responses and identification of salt tolerance genes in a chromosome segment substitution line of 'Khao dawk Mali 105' rice. Environ Exp Bot. 2018;155(1):497–508.

Cirillo V, Molisso D, Aprile AM, Maggio A, Rao R. Systemin peptide application improves tomato salt stress tolerance and reveals common adaptation mechanisms to biotic and abiotic stress in plants. Environ Exp Bot. 2022;199(1):104865.

Edwards K, Johnstone C, & Thompson C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 1991;19(6):1349.

Habila S, Khunpolwattana N, Chantarachot T, Buaboocha T, Comai L, Chadchawan S, Pongpanich M. Salt stress responses and SNP-based phylogenetic analysis of Thai rice cultivars. Plant Genome. 2022;15(1):e20189.

Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G. GSDS 2.0: An upgraded gene feature visualization server. Bioinformatics. 2015;31(8):1296–1297.

Jackson MT. Conservation of rice genetic resources: The role of the International Rice Genebank at IRRI. Plant Mol Biol. 1997;35(1):61–67.

Kanstrup C, Nour-Eldin HH. The emerging role of the nitrate and peptide transporter family: NPF in plant specialized metabolism. Current Opinion in Plant Biology. 2022; 68: doi:10.1016/j.pbi.2022.102243

Katawatin R, Sukchan S. Mapping soil salinity and soil erosion in Thailand with emphasis on computer-assisted techniques. Pedologist. 2012; 55(3): 343–354.

Kojonna T, Suttiyut T, Khunpolwattana N, Pongpanich M, Suriya-arunroj D, Comai L, Buaboocha T, Chadchawan S. Identification of a negative regulator for salt tolerance at seedling stage via a Genome-wide association study of thai rice populations. Int J Mol Sci. 2022;23(3): doi:10.3390/ijms23031842

Li Y, Ouyang J, Wang YY, Hu R, Xia K, Duan J, Wang Y, Tsay YF, Zhang M. Disruption of the rice nitrate transporter *OsNPF2.2* hinders root-to-shoot nitrate transport and vascular development. Sci Rep. 2015;5(1):9635.

Liu C, Mao B, Yuan D, Chu C, Duan M. Salt tolerance in rice: Physiological responses and molecular mechanisms. Crop J. 2022;10(1):13–25.

Longo A, Miles NW, Dickstein R. Genome Mining of Plant NPFs Reveals Varying Conservation of Signature Motifs Associated with the Mechanism of Transport. Front Plant Sci. 2018; 871:1668.

Murashige T, Skoog FK. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15:473–497.

Nakaminami K, Okamoto M, Higuchi-Takeuchi M, Yoshizumi T, Yamaguchi Y, Fukao Y, Shimizu M, Ohashi C, Tanaka M, Matsui M *et al*. *AtPep3* is a hormone-like peptide that plays a role in the salinity stress tolerance of plants. Proc Natl Acad Sci U S A. 2018;115(22):5810–5815.

Ouyang J, Cai Z, Xia K, Wang Y, Duan J, Zhang M. Identification and analysis of eight peptide transporter homologs in rice. Plant Sci. 2010;179(4):374–382.

Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, Childs K, Thibaud-Nissen F, Malek RL, Lee Y, Zheng L, Orvis, *et al*. The TIGR Rice Genome Annotation Resource: Improvements and new features. Nucleic Acids Res. 2007;35(1):D883-D887.

Pao SS, Paulsen IT, Saier MHJ. Major facilitator superfamily. Microbiol Mol Biol Rev. 1998;62(1):1–34.

Park HJ, Seo BS, Jeong YJ, Yang HI, Park S, Baek N, Kwak JH, Choi WJ. Soil salinity, fertility and carbon content, and rice yield of salt-affected paddy with different cultivation period in southwestern coastal area of South Korea. Soil Sci Plant Nutr. 2022;68(1):53–63.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res, 2001;29(9):e45.

Prabhala BK, Rahman M, Nour-eldin HH, Jørgensen FS, Mirza O. Chapter Ten—PTR2/POT/NPF transporters: What makes them tick? In R. Donev (Eds). Adv Protein Chem Struct Biol. Academic Press, Amsterdam; 2021. p. 219–240.

Pusadee T, Jamjod S, Chiang Y-C, Rerkasem B, & Schaal BA. Genetic structure and isolation by distance in a landrace of thai rice. Proc Natl Acad Sci USA. 2009;106(33):13880-13885.

Sonsungsan P, Chantanakool P, Suratanee A, Buaboocha T, Comai L, Chadchawan S, Plaimas K. Identification of Key Genes in “Luang Pratahn”, Thai Salt-Tolerant Rice, Based on Time-Course Data and Weighted Co-expression Networks. Front Plant Sci. 2021;12:744654.

Steiner HY, Song W, Zhang L, Naider F, Becker JM, Stacey G. An Arabidopsis peptide transporter is a member of a new class of membrane transport proteins. Plant Cell. 1994;6(9):1289–1299.

Wellburn AR. The Spectral Determination of Chlorophylls *a* and *b*, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. J Plant Physiol. 1994;144(3):307–313.

Xu D, Zhang Y. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. Biophys J. 2011;101(10):2525–2534.

Yang X, Xia X, Zeng Y, Nong B, Zhang Z, Wu Y, Tian Q, Zeng W, Gao J, Zhou W, *et al*. Genome-wide identification of the peptide transporter family in rice and analysis of the PTR expression modulation in two near-isogenic lines with different nitrogen use efficiency. BMC Plant Biol. 2020;20(1):193.

Zhao X, Huang J, Yu H, Wang L, Xie W. Genomic survey, characterization and expression profile analysis of the peptide transporter family in rice (*Oryza sativa* L.). BMC Plant Biol. 2010;10(1):92.

Supplementary Table 1 Peptide transport genes annotated in Rice Genome Annotation Project (Ouyang *et al*., 2007).

Chr	Locus Identifier	Putative Function
Chr1	LOC_Os01g01360	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g04950	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g37590	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g54515	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g55600	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g55610	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g65100	peptide transporter, putative, expressed
Chr1	LOC_Os01g65120	peptide transporter, putative, expressed
Chr1	LOC_Os01g65130	peptide transporter, putative, expressed
Chr1	LOC_Os01g65140	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g65190	POT domain containing peptide transporter, putative, expressed
Chr1	LOC_Os01g67630	peptide transporter PTR2, putative, expressed
Chr1	LOC_Os01g67640	PTR-like peptide transporter, putative, expressed
Chr1	LOC_Os01g68330	antigen peptide transporter-like 1, chloroplast precursor, putative, expressed
Chr1	LOC_Os01g68510	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02080	PTR-like peptide transporter, putative, expressed
Chr10	LOC_Os10g02100	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02210	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02220	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02240	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02260	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g02340	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g05780	POT domain containing peptide transporter, putative, expressed
Chr10	LOC_Os10g22560	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g33170	POT domain containing peptide transporter, putative, expressed
Chr10	LOC_Os10g33210	peptide transporter PTR3-A, putative, expressed
Chr10	LOC_Os10g40600	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g42870	peptide transporter PTR2, putative, expressed
Chr10	LOC_Os10g42900	peptide transporter PTR2, putative, expressed
Chr11	LOC_Os11g12740	peptide transporter PTR2, putative, expressed
Chr11	LOC_Os11g18044	peptide transporter PTR2, putative, expressed
Chr11	LOC_Os11g23890	peptide transporter PTR2, putative, expressed
Chr12	LOC_Os12g12934	peptide transporter PTR3-A, putative, expressed
Chr12	LOC_Os12g44100	peptide transporter PTR2, putative, expressed
Chr2	LOC_Os02g46460	peptide transporter PTR2, putative, expressed
Chr2	LOC_Os02g47090	peptide transporter PTR2, putative, expressed
Chr2	LOC_Os02g48570	peptide transporter PTR2, putative, expressed
Chr2	LOC_Os02g52060	peptide transporter like protein, putative, expressed
Chr3	LOC_Os03g01290	peptide transporter PTR2, putative, expressed
Chr3	LOC_Os03g04570	peptide transporter PTR3-A, putative, expressed
Chr3	LOC_Os03g13240	peptide transporter, putative, expressed
Chr3	LOC_Os03g13250	peptide transporter PTR2, putative, expressed
Chr3	LOC_Os03g13274	peptide transporter PTR2, putative, expressed

Chr	Locus Identifier	Putative Function
Chr3	LOC_Os03g48180	peptide transporter PTR2, putative, expressed
Chr3	LOC_Os03g51050	peptide transporter PTR2, putative, expressed
Chr3	LOC_Os03g54880	antigen peptide transporter-like 2, putative, expressed
Chr3	LOC_Os03g60850	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g36040	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g39030	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g41400	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g50930	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g50940	peptide transporter PTR2, putative, expressed
Chr4	LOC_Os04g50950	peptide transporter PTR2, putative, expressed
Chr5	LOC_Os05g27010	peptide transporter PTR3-A, putative, expressed
Chr5	LOC_Os05g27050	peptide transporter, putative, expressed
Chr5	LOC_Os05g27304	peptide transporter PTR2, putative, expressed
Chr5	LOC_Os05g33960	peptide transporter PTR2, putative, expressed
Chr5	LOC_Os05g34010	peptide transporter PTR2, putative, expressed
Chr5	LOC_Os05g34030	peptide transporter PTR2, putative, expressed
Chr5	LOC_Os05g35594	peptide transporter, putative, expressed
Chr5	LOC_Os05g35650	peptide transporter PTR2, putative, expressed
Chr6	LOC_Os06g13210	peptide transporter PTR2, putative, expressed
Chr6	LOC_Os06g15370	peptide transporter PTR2, putative, expressed
Chr6	LOC_Os06g38294	peptide transporter PTR2, putative, expressed
Chr6	LOC_Os06g49220	peptide transporter, putative, expressed
Chr6	LOC_Os06g49230	peptide transporter, putative, expressed
Chr6	LOC_Os06g49240	PTR-like peptide transporter, putative, expressed
Chr6	LOC_Os06g49250	peptide transporter PTR2, putative, expressed
Chr7	LOC_Os07g01070	peptide transporter, putative, expressed
Chr7	LOC_Os07g41250	peptide transporter PTR2, putative, expressed
Chr8	LOC_Os08g05910	peptide transporter PTR2, putative, expressed
Chr8	LOC_Os08g41590	peptide transporter PTR2, putative, expressed
Chr8	LOC_Os08g41600	peptide transporter, putative, expressed