Expression and characterization of rice putative \textit{PAUSED} gene

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In \textit{Arabidopsis}, \textit{PAUSED} (PSD) encodes the ortholog of los1p/exportin-t, which mediates the nuclear export of transfer RNA (tRNA) in yeast and mammals. However, in monocot plants such as rice, knowledge of the corresponding ortholog is limited, and its effects on growth development and productivity remain unknown. In this study, we verified a rice transfer-DNA insertional mutant \textit{psd} line and analyzed its phenotypes; the mutant displayed severe morphological defects including retarded development and low fertility compared with wild-type rice. Examining intronless \textit{tRNA-Tyr} and intron-containing pre-\textit{tRNA-Ala} expression levels in cytoplasmic and nuclear fraction with Northern blot analysis between wild-type and mutant leaf tissue suggested that rice PSD might be involved in tRNA export from the nucleus to the cytoplasm. Additionally, reverse transcription-polymerase chain reaction analysis revealed that \textit{PSD} transcript was expressed throughout normal rice plant development, and subcellular localization assays showed that rice PSD protein was present in both the nucleus and cytoplasm. In summary, our data implied that the putative \textit{PSD} gene might be indispensable for normal rice development and its function might be the same as that of \textit{Arabidopsis} PSD.

Keywords: \textit{PAUSED}; tRNA export; development defects; rice

The transport of different RNA species from the nucleus to the cytoplasm is fundamental to gene expression. A general paradigm has been established in which RNA are exported through the nuclear pore complexes via mobile export receptors after being produced in the nucleus. Small RNA, such as transfer RNA (tRNA) and microRNA, follow a relatively simple pathway by directly binding to export receptors, while large RNA, such as rRNA and mRNA, are assembled into complicated ribonucleoprotein with exporters or other specific adaptor proteins [1,2].

As a member of the karyopherin superfamily, exportin-t is the principal tRNA exporter in vertebrate cells that binds to tRNA in a typical Ran-GTPase-dependent manner. Properly processed 5$'$ and 3$'$ termini, secondary and tertiary structural elements of tRNA, are decoded by exportin-t, meaning that tRNA quality control is performed before export [3–5]. In plants, \textit{Arabidopsis} PSD is the ortholog of exportin-t and may function as tRNA exporter [6]; the \textit{psd} mutant displays a series of growth and development defects, including delayed leaf production and abnormal inflorescence morphology. Rice is a model monocot plant that differs from \textit{Arabidopsis}, which is representative of dicot plants. The expression pattern of \textit{PSD} homolog and its role in normal rice plant development remain unknown. In this study, we employed a rice transfer-DNA (T-DNA) insertion mutant to explore rice \textit{PSD} to gain a more comprehensive understanding of plant \textit{PSD} gene annotation and to verify the diversity and complexity of pathways for plant tRNA export [7].

Materials and Methods

\textbf{Plant culture}

Wild-type rice (\textit{Oryza sativa} L. subsp. Japonica cv. Dongjin) and T2 generation T-DNA insertional line (No. 1B-01410) seeds were obtained from Plant Functional Genomics Laboratory (Kyoungbuk, Korea) [8–11]. The
Sequence annotation and phylogenetic analysis
PSD gene sequences were downloaded from the NCBI gene database. Exons were indicated with Vector NTI (Invitrogen, Carlsbad, USA). Protein sequences showing obvious identity were downloaded from the rice PSD BLAST result in the NCBI protein database (http://www.ncbi.nlm.nih.gov/blast). Sequences were aligned using ClustalX1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) and further adjusted manually in BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) [12,13]. Phylogenetic analysis was done using the maximum likelihood method by tree-puzzle-5.2 (http://www.tree-puzzle.de/) [14].

Polymerase chain reaction (PCR) verification of the T-DNA insertional mutant
Leaf genomic DNA was purified using traditional CTAB method [15]. Two common PCR were performed for the differentiation of wild-type, heterozygote and homozygote plants. The primers were designed using the NCBI database and web service (http://signal.salk.edu/cgi-bin/RiceGE). Primers for rice genome (PCR product: 409 bp) are 5’-TGCAGCTTTATTTTCAATTA-3’ (forward) and 5’-GGGTAGTTGCTGAAGATTTA-3’ (reverse); primers for the T-DNA genome (PCR product: 543 bp) are 5’-TCAGCCCATTGATCATAGT-3’ (forward) and 5’-CGCTGAGAATTTAGCACC-3’ (reverse). In brief, the PCR was conducted for 30 cycles in a thermal controller (PTC-100; Bio-Rad). Each amplification cycle consisted of 0.5 min at 94 ºC for denaturizing, 0.5 min at 55 ºC for primer annealing and 1 min at 72 ºC for extension.

Reverse transcription (RT)-PCR assay
Total RNA was extracted with TRizol reagent (Invitrogen, Carlsbad, USA). Initially, 2 µg total RNA was reverse transcribed with gene-specific downstream primer by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA) in a total volume of 25 μl, and cDNA was generated at 37 ºC for 30 min. Expression level was determined using Taq PCR MasterMix system (Tiangen, Beijing, China) in a total volume of 25 μl reaction mixture containing 12.5 μl 2× master mix, 0.5 μl (20 μM) sense primer, 0.5 μl (20 μM) antisense primer, 2 μl cDNA template and 9.5 μl distilled water. The PCR was then performed for 28 amplification cycles in the thermal controller (PTC-100; Bio-Rad). Each cycle consisted of 0.5 min at 94 ºC for denaturizing, 0.5 min at 56 ºC for primer annealing and 1 min at 72 ºC for extension. Primers were used as follows: Actin (PCR product: 336 bp): 5’-TCCATCTGCTCATCTCAG-3’ (forward) and 5’-GT-ACCCCTCATCGCATCTG-3’ (reverse); PSD (PCR product: 126 bp): 5’-CGACAGTTGCTGAT-3’ (forward) and 5’-TGCGATGCAGAAGACC-3’ (reverse).

Subcellular localization assay
The full coding sequence used for transient expression of PSD in onion epidermal cells was amplified from full length cDNA clone AK099895 using the forward primer 5’-GGATCCCGACGACCTCGAGCAGGCCAT-3’ and reverse primer 5’-GAGCTCTCATCTGAGAAGACACTG-3’. The PCR product was subcloned into the BamHI/SalI site of pA7-green fluorescent protein (GFP) expression vector under control of the enhanced cauliflower mosaic virus 35S promoter. The recombinant pA7-GFP-PSD plasmid and pA7-GFP plasmid (used as control) were separately transferred by bombardment into onion epidermal cells using a gene gun (PDS-1000/He; Bio-Rad) according to the instruction manual. Transformed cells were examined using a confocal microscope (Olympus FV500, Tokyo, Japan) after incubation at 25 ºC for 24 h on Murashige and Skoog medium.

Cellular fractionation
Cellular fractionation was performed as described [6]. Briefly, leaf tissue from 4-week-old seedings were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Cell wall-disrupting buffer [10 mM potassium phosphate (pH 7.0), 100 mM NaCl, 10 mM 2-mercaptoethanol, 1 M hexylene glycol] was then added to make a thick slurry. This mixture was filtered through Miracloth (Calbiochem, San Diego, USA) to remove large chunks of tissue and centrifuged at 1500 g for 10 min at 4 ºC to pellet nuclei and cell debris. After centrifugation, the supernatant was collected and recentrifuged at 13,000 g for 15 min at 4 ºC. The supernatant of this second centrifugation was saved for the cytoplasmic fraction. The first pellet was washed with nuclei preparation buffer [10 mM potassium phosphate (pH 7.0), 100 mM NaCl, 10 mM 2-mercaptoethanol, 1 M hexylene glycol, 10 mM MgCl2, 0.5% Triton X-100] and centrifuged at 1500 g for 10 min at 4 ºC. After centrifugation, the supernatant was discarded and the pellet was washed with nuclei preparation buffer. Washing and centrifugation were repeated four to five times, and the final pellet was saved for the nuclear fraction. RNA was extracted from the cytoplasmic and nuclear fractions.
with TRIzol reagent [16].

**Northern blot analysis for tRNA**

A total of 10 μg cytoplasmic and 2 μg nuclear RNA were subjected to electrophoresis on an 8 M urea/12% denaturing polyacrylamide gel, transferred to Hybond N+ membranes (Amersham Pharmacia, Uppsala, Sweden) and then hybridized with \([\gamma^{32}\text{P}]-\)labeled probes. Oligonucleotide probes were labeled with T4 polynucleotide kinase (MBI Fermentas, St Leon-Rot, Germany). Hybridization and washing were carried out at 40 °C in the ExpressHyb Hybridization Solution (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions. The membrane was air-dried and then measured by phosphorimager (Amersham Pharmacia). 5S rRNA served as the loading control for the quantitative calculation, and U6 small nuclear RNA was used as marker of nuclear RNA. Probe sequences were as follows: 5S rRNA: 5′-AGGACCTCCAGAGGTACC-3′; U6 RNA: 5′-TCGATTTGTGGGTCAAT-3′; tRNA-tyr: 5′-ACCTGGAGTCCGAAACCAACGCG-3′; pre-tRNA-Ala: 5′-CAAGAAATGGAGATTCAACCC-3′.

**Results**

**Genomic organization of rice putative **PSD**

Los1p/exportin-t proteins form a large family among many species, such as yeast, animals and plants, containing xpo1 domain in the primary structure [17,18]. By searching the rice genome database, we found that only one homolog’s existence was previously validated by rice full-length database, and we then sketched a phylogenetic tree. As expected, rice PSD is most identical to *Arabidopsis* PSD and the degree of similarity depends on the distance between the species [Fig. 1(C)]. Together with other homologs from human, mice and so on, they form a clade with high bootstrap support. Additionally, proteins from fungi form another independent clade. Reasonably, all these proteins come from eukaryotes, indicating that the origin of these homologs possibly occurred after the segregation of eukaryote and prokaryote.

**Rice **PSD** was expressed throughout the development in normal plant**

In order to study the general spatio-temporal expression pattern of **PSD** in normal rice plants, we subsequently examined the expression of **PSD** in various tissues from different phases of wild-type rice by RT-PCR. As expected, **PSD** was expressed throughout the development process [Fig. 2(A)], which is consistent with the fundamental nature of its possible role in tRNA export.

**Subcellular localization of the rice **PSD** protein in onion epidermal cells**

Transient expression of **GFP** gene fusion in onion epidermis is a credible method for the investigating subcellular localization of GFP protein in plant cells [20]. We used this method to study the subcellular localization of rice **PSD**, and results showed that GFP and GFP-**PSD** were both distributed throughout the cells [Fig. 2(B)], implying that **PSD** shuttle between nucleus and cytoplasm to perform its function.

**Knockout of rice putative **PSD** transcript by T-DNA insertion caused severe development defects**

To further investigate rice **PSD** function, we obtained T2 generation T-DNA insertional mutant line (1B-01410) seeds from Plant Functional Genomics Laboratory to insert within the gene [Fig. 3(A)] [10,11]. Thirty-one homozygote plants were chosen with common PCR reaction. The wild-type and heterozygote plants showed a sharp band (409 bp) with genomic forward and reverse primers designed around the insertion site, while the homozygote plant exhibited a negative result. Another pair of T-DNA primers was used to differentiate heterozygote from wild-type plants;
Fig. 1 Phylogenetic analysis of PAUSED (PSD) homologs  (A) Schematic representation of rice PSD gene organization and protein structure. The light orange arrows stand for the exons in the genome. Xpo1 domain was indicated in red and exists in almost all PSD homologs. The blue part, the putative tRNA interaction region, was assumed to be necessary in tRNA interaction and nuclear export according to function annotation of the corresponding region in human PSD. (B) Phylogenetic analysis of PSD homologs across various eukaryotes. ScLOS1 was used as the out group control. Ac, *Aspergillus clavatus*; An, *Aspergillus niger*; Ao, *Aspergillus oryzae*; AtPSD, *Arabidopsis thaliana*; AtXPOT, *Aspergillus terreus*; Ce, *Caenorhabditis elegans*; Cg, *Candida glabrata*; Ci, *Coccidioides immitis*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nf, *Neosartorya fischeri*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; XPOT, los1p/exportin-t.
19 heterozygote plants showed an obvious PCR product of 543 bp while none of wild type did [Fig. 3(B)]. To confirm the knockout of PSD transcript in homozygote, we examined the PSD expression in leaf tissue with RT-PCR, and results revealed that T-DNA insertion completely disrupted the PSD expression [Fig. 3(C)].

Nearly all the homozygote plants displayed development retardation, such as delayed panicle heading and reduced size in the first several development stages, compared with the wild type [Fig. 3(D), upper]. These results are not so obvious for the Arabidopsis psd mutant possibly because of the different mutant pattern or the deviation of plant species. When the plants grew mature, their height and appearance were almost indiscernible [Fig. 3(D), bottom]. However, for plants losing PSD function, the seed-setting rate decreased significantly [Fig. 3(E,F)], as was nearly the case for Arabidopsis psd mutant [21]. Taken together, loss of function of PSD severely impaired the normal rice development process, and the most notable sharing phenotype with Arabidopsis psd mutant implies that the functions of the homologs are coupled.

Rice PSD might regulate the tRNA export in vivo

To examine the role of rice PSD in tRNA export, we examined the distribution of intronless tRNA-Tyr and intron-containing pre-tRNA-Ala (GGC) in the nuclear and
Rice PAUSED function annotation

Fig. 3 Loss of function of rice PAUSED (PSD) with T-DNA insertion caused development defects  (A) Display of molecular basis for rice T-DNA insertional mutant line 1B-01410. (B) A sketch of two common PCR results for heterozygote, homozygote and wild-type rice plant. P1 and P2 showed the positive PCR product. N showed a negative result. (C) Reverse transcription-PCR confirmation of the non-existence of PSD transcript in homozygote mutant compared to the presence in wild-type (leaf tissue selected). P, PSD homozygote mutant line; W, wild type. (D) Appearance of wild type and rice psd mutant line about 10-week old (upper) and in the mature stage (bottom). (E) Comparative views of typical wild-type (WT) and psd mutant line seeds. (F) Graphical presentation of the seed setting rate (number of fertile grains/ number of total grains) between wild-type (W) and psd (P) mutant line. Results are presented as mean±SD. For statistical analysis, Student’s t-test was used. Difference was considered significant at a level of P<0.05.

cytoplasmic fractions of leaf tissue from wild-type and psd double mutant with Northern blot analysis. As anticipated, the mutant slightly decreased the level of both tRNA in the cytoplasm and increased their levels in the nucleus. We were particularly interested in the mutation’s effect on pre-tRNA-Ala because accumulation of unspliced tRNA can be used to diagnose defects in tRNA export in yeast. Moreover, as the Arabidopsis psd mutant increased
the level of unspliced tRNA-Tyr [22], the much higher level of pre-tRNA-Ala in the nucleus of rice psd should provide evidence of its regulatory role in tRNA export in vivo (Fig. 4).

In Arabidopsis, PSD transcript was observed in a series of tissues such as roots, vegetative leaves, floral buds, shoot apex and so on. Its omnipresence was also concluded in rice thus we did not quantitatively measure the expression level. Similar omnipresence was also concluded in Arabidopsis. RT-PCR application merely amplified part of the PSD transcript, so we could not exclude the possibility that PSD was differentially spliced in some tissue regions that we assayed. We also could not rule out the transcriptional regulation of PSD at a tissue- or cell-specific level. Further study of PSD regulation should increase our understanding of its role in tRNA export pathways and its function in rice development.

Inhibition of tRNA nuclear exporter in yeast and Arabidopsis caused the accumulation of intron-containing pre-tRNA, leading to the prediction that pre-tRNA splicing could occur in the cytoplasm [22], and the case is the same for rice. Based on the expression and characterization of rice PSD, we proposed a simple hypothetical model for the involvement of PSD in the process of tRNA nuclear export. Intronless and intron-containing pre-tRNA were transcribed, processed, edited in the nucleus and then exported to the cytoplasm in the presence of PSD and other associated factors through the nuclear pore complex. In the cytoplasm, the tRNA cargo was released for later splicing (for pre-tRNA), translation or aminoacylation.

If rice PSD indeed functions as tRNA exporter in vivo, we speculated that it might not be the only tRNA export receptor in rice; though there were no other homologs according to BLAST up-to-date rice genome database. Also, notably, null allele of this gene was viable. Actually, Arabidopsis psd mutant slightly increased the level of intronless tRNA-Met in the nucleus, but it did not affect the cytoplasmic level of this tRNA [6], which demonstrated that it is not absolutely required for this process. Based on sequence alignment and conservation analysis, we tentatively annotated the function domain of rice PSD. However, it was rather difficult to predict the in vivo interaction mechanism due to a lack of a consistent in vitro assay to recreate events that occur during tRNA

Discussion

PSD is a member of the importin β family of nucleocytoplasmic transport receptors. It is hard to predict the role for this superfamily. For example, exportin 5, the mammalian ortholog of HASTY (HST) and another member of importin β family, exports pre-microRNA, tRNA, a viral hairpin RNA and proteins associated with these and other double-stranded RNA, while HST’s yeast ortholog, Msn5p, exports several different types of phosphorylated proteins and imports replication protein A [23]. Though rice PSD shares 51% sequence identity with Arabidopsis PSD, the experimental validation is much more persuasive and necessary to support the idea that they share the same function. The ever-growing populations of T-DNA insertions in rice represent such a powerful tool for study of gene function. In this study, we verified a rice T-DNA insertional mutant psd and substantiated the function assignment of rice PSD. The phenotypes of rice psd mutant were not investigated in detail and other useful clues may have been missed. These phenotypes are possibly caused by the loss of function of rice PSD. An efficient way to confirm this point would be to construct the over-expressed RNA interference vector to perform the complementation test, and then observe the phenotypes of the transgenic plants. Considering that rice PSD is the homolog of Arabidopsis PSD that provided our biochemical data about the mutant, we assumed that these phenotypes likely originated from the impairment of tRNA export and the resultant translation limitation, which might elucidate rice PSD function to some extent.

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generation, processing and RNA-protein assembly, especially in plants [24]. Moreover, we do not have a profound structural knowledge of tRNA export. Deciphering the complicated interaction between PSD with tRNA is therefore still a major challenge [25–27].

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