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Isolation and characterization of a new *DUR3*-like gene, *PyDUR3.3*, from the marine macroalga *Pyropia yezoensis* (Rhodophyta)

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Abstract

DUR3 proteins mediate high-affinity transport of exogenous and endogenous urea. Although two *DUR3*-like genes (*PyDUR3.1/3.2*) have been identified in *Pyropia yezoensis*, a BLAST search using these sequences against the *P. yezoensis* EST database suggested the existence of another *DUR3*-like gene (*PyDUR3.3*). In this study, the *PyDUR3.3* gene was isolated and characterized and compared to *PyDUR3.1/3.2* genes. The predicted length of *PyDUR3.3* was 679 amino acids, which included 15 transmembrane domains. An amino acid sequence alignment of algal, plant, and yeast *DUR3* proteins showed that *PyDUR3.3* was more similar to *PyDUR3.2* than to other *DUR3* proteins including *PyDUR3.1*. Exon-intron structures of *PyDUR3.3* and *PyDUR3.2* were also closely related to each other, which clearly differed from that of *PyDUR3.1*. Expression analysis showed that *PyDUR3.3* mRNA levels were extremely high in sporophytes, regardless of the nutrient condition, compared to gametophytes. On the other hand, expression of *PyDUR3.2* and *PyDUR3.1* was high in the gametophytes and sporophytes, respectively, and induced by nutrient starvation. These results suggest that expression of *PyDUR3.3/3.2/3.1* depends on the life history phase as well as the nutrient conditions, and that *PyDUR3.3* and *PyDUR3.2* are paralogues specifically differentiated in function and life history phase.

Keywords: cDNA cloning • *DUR3* • gene expression • *Pyropia yezoensis* • Rhodophyta • sodium solute symporter • urea transporter

Introduction

The rhodophyte genus *Pyropia* represents a unique heteromorphic and digenetic life cycle that consists of a leafy gametophyte and a filamentous sporophyte [1]. These developmental generations, in which *Pyropia* species form a thallus and a conchocelis, respectively, show differences in physiological and structural features, such as optimum growth conditions, cell wall composition, and chloroplast and chromosome numbers [2–7]. Since the heteromorphic life cycle can be reproduced within a few months in the laboratory, *P. yezoensis* has been utilized as a valuable model organism for fundamental and applied marine macroalgal research [5, 8].

In Japan, *P. yezoensis* is one of the most important algal species used as food, and approximately 350,000 tons (wet weight) of the gametophytic thalli are produced in *Pyropia* cultivation farms every year, most of which are processed to dried sheets of “*nori*” (e-Stat, portal site of official statistics of Japan: <http://www.e-stat.go.jp/SG1/estat/eStatTopPortal.do> “Accessed 24 Jun 2015”). The yield and quality of the cultivated *Pyropia* thalli are frequently affected by environmental factors. In particular, growth, development, and quality of *Pyropia* thalli are influenced by changing dissolved inorganic nitrogen (DIN) content, which consists of nitrate-N ($\text{NO}_3\text{-N}$), nitrite-N ($\text{NO}_2\text{-N}$), and ammonium-N ($\text{NH}_4\text{-N}$) [9–11]. Although these inorganic-N sources are essential for growth and development in photosynthetic eukaryotes, some plants and algae, including *Pyropia*, can efficiently utilize not only these types of inorganic-N but also organic-N such as urea-N and amino acid-N (AA-N) [9, 12–18]. In order to investigate the molecular mechanisms for inorganic/organic-N uptake and assimilation in *Pyropia* thalli, a subtracted cDNA library was constructed from *P. yezoensis* thalli grown in different nutrient conditions, and transcripts differentially expressed have been analyzed. To date, three *P. yezoensis* genes, a nitrate transporter gene, *PyNRT2*, and two urea transporter genes, *PyDUR3.1* and *PyDUR3.2* (previously designated as *PyUT1* and *PyUT2*, respectively), which may be involved in uptake and transport of nitrate and urea, respectively, have been identified [19]. In *P. yezoensis* thalli, the transcripts of both *PyDUR3* genes were markedly up-regulated under N-deficient conditions, suggesting that these proteins are associated with N-acquisition pathways for the direct utilization of urea from the environment.

DUR (Degradation of URea) genes were first characterized in yeasts. In the yeast *Saccharomyces cerevisiae*, the cytoplasmic enzymes urea carboxylase (*DUR1*) and allophanate hydrolase (*DUR2*), catalyzing degradation of urea to ammonia and carbon dioxide, were originally considered to be two separate genes, but were later found to be encoded by a single polypeptide and thus renamed *DUR1,2* [20, 21]. The *DUR3* gene family encodes membranous urea transporter proteins which are structurally related to the sodium solute symporter (SSS) superfamily. SSS proteins are integral membrane proteins containing 11 to 15 putative transmembrane domains (TDs), which mediate the transport of wide variety of substrates (e.g., AAs, inositols, sugars, and urea) across cytoplasmic membranes [16, 18, 22]. In photosynthetic eukaryotes, *AtDUR3* from the model land plant *Arabidopsis thaliana* has been well characterized. *AtDUR3* (previously named At5g54380) was identified as a putative urea transporter gene by a genome-wide *in silico* search using various urea transporter sequences from mammals and microbes against the *Arabidopsis* genome, showing approximately 40% sequence identity to the yeast functional urea transporter gene, *S. cerevisiae* *ScDUR3* [23, 24]. *AtDUR3* is expressed in roots, shoots, and leaves, and up-regulated under N-deficiency in roots, during early germination in seeds and leaf senescence, indicating that *AtDUR3* provides pathways not only for direct uptake of soil urea but also for retranslocation or recycling of endogenous urea [24–26]. In addition, genome sequence analysis indicates that model land plants including *A. thaliana* have only a single *DUR3* gene [18, 24, 25, 27–29]. On the other hand, two *DUR3* genes have been identified in some algal species, including *P. yezoensis* [19, 30, 31], although the molecular characteristics remain unclear.

In *P. yezoensis*, a large-scale expressed sequence tag (EST) analysis has been carried out and an EST database, including approximately 10,600 and 10,200 EST sequences from the gametophytic and sporophytic generations, respectively, has been made available [32, 33]. Interestingly, a BLAST search using *PyDUR3.1* and *PyDUR3.2* cDNA nucleotide (nt) sequences [19] against the EST database suggested the possibility that *P. yezoensis* possesses another *DUR3*-like gene (*PyDUR3.3*) predominantly expressed in the sporophytic generation. The present study was undertaken to clone and sequence the *P. yezoensis* *PyDUR3.3* cDNA and genomic DNA (gDNA). In addition, in order to understand the differences in physiological and molecular characteristics among the three *PyDUR3* genes, *PyDUR3.3*,

PyDUR3.2, and *PyDUR3.1*, their sequence similarities, genomic organization, and level of transcript accumulation between the heteromorphic generations were analyzed.

Materials and methods

Materials and cultivation conditions

Pyropia yezoensis (strain FA-89), which was originally isolated by selective breeding and maintained by the Fukuoka Fisheries and Marine Technology Research Center, Japan [34, 35], was used in this study. Leafy gametophytes (thalli) and filamentous sporophytes (conchocelis) of the *P. yezoensis* were maintained using one-fifth strength Provasoli's enriched seawater (1/5 PES) medium [36] in the laboratory. The culture medium was changed every 2 days for the thalli and 7 days for the conchocelis throughout the experiments. The thallus culture was aerated with air filtered through a FP30/0.2PTFE-S filter (Whatman, Dassel, Germany), irradiated with 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ light on a 10:14 h (light/dark) cycle (10L/14D photoperiod), and maintained at 10°C. The conchocelis culture was maintained statically, irradiated with 10 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ light on a 14L/10D photoperiod at 18°C. Thalli and conchocelis cultures were inoculated into the experimental seawater medium upon reaching an average length of 3 cm or an average wet weight of 100 mg, respectively.

Dissolved inorganic nitrogen (DIN) content in natural seawater for preparation of each experimental seawater medium was analyzed using a TRAACS 2000 (Bran+Luebbe, Norderstedt, Germany). The average DIN concentration of the natural seawater was 257.6 $\mu\text{g/L}$ ($\text{NO}_3\text{-N}$ = 240.4 $\mu\text{g/L}$, $\text{NO}_2\text{-N}$ = 4.5 $\mu\text{g/L}$, and $\text{NH}_4\text{-N}$ = 12.7 $\mu\text{g/L}$).

Total RNA and gDNA extraction

Total RNA extraction from fresh *P. yezoensis* thalli and conchocelis was performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). After DNase treatment using the MessageClean Kit (GenHunter, Tennessee, USA), the purified total RNA was stored at -80°C until single-stranded (ss) cDNA synthesis

for 3' or 5' rapid amplification of cDNA ends (RACE) and quantitative-polymerase chain reaction (qPCR) was performed.

Genomic DNA (gDNA) extraction from fresh *P. yezoensis* thalli was performed according to a previously-reported method [37, 38]. The extracted gDNA was further purified using the DNeasy Plant Mini Kit (Qiagen). The gDNA obtained was used for isolation of partial gDNA clones of *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1*.

PyDUR3.3 cDNA cloning

Initially, for isolation of *P. yezoensis PyDUR3.3* cDNA fragments, 3' RACE was used. Five micrograms of total RNA were used for ss cDNA synthesis using the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, California, USA). The PCR amplification of *PyDUR3.3* cDNA was performed using gene-specific primer *PyDUR3.3-EP1*, which was designed based on the nt sequences of ESTs AU192239 (542 bp) and AU194122 (490 bp) in the *P. yezoensis* EST database (*Porphyra* EST Index, Kazusa DNA Res. Inst., Chiba, Japan: <http://est.kazusa.or.jp/en/plant/porphyra/EST/> “Accessed 24 Jul 2013”) [32, 33], and the adapter primer AUAP from the kit (Table 1). The cDNA fragment amplified by PCR was subcloned into a pT7Blue T-vector (Novagen, Wisconsin, USA) and sequenced. Subsequently, gene-specific primers for *PyDUR3.3* were synthesized for 5' RACE.

In order to determine the 5' end of the *PyDUR3.3* cDNA, the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen) was used. Single-stranded cDNA for 5' RACE was synthesized from 5 µg of total RNA using the gene-specific primer *PyDUR3.3-SP1* (Table 1). After dC-tail addition to the 3' end of the cDNA, the PCR amplification of the dC-tailed cDNA was performed using the gene-specific primer *PyDUR3.3-SP2* (Table 1) and the adapter primer AAP from the kit. The nested PCR amplification was performed using a set of the gene-specific primer *PyDUR3.3-SP3* and the adapter primer AUAP from the kit (Table 1), and the amplified cDNA fragments were subcloned into a pT7Blue T-vector and sequenced.

PyDUR3.3, *PyDUR3.2*, and *PyDUR3.1* gDNA cloning

Genomic copies of *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* was amplified by Long and Accurate-PCR (LA-PCR, Takara Bio, Kyoto, Japan) according to the supplier's instructions using three sets of gene-specific primers, *PyDUR3.3*-GF/GR for *PyDUR3.3*, *PyDUR3.2*-GF/GR for *PyDUR3.2*, and *PyDUR3.1*-GF/GR for *PyDUR3.1* (Table 1), synthesized based on the nt sequences of the *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* cDNAs, respectively. The gDNA fragments amplified by LA-PCR were subcloned into a pT7Blue T-vector and sequenced.

Sequence analysis

Plasmid DNAs including cDNA or gDNA fragments were isolated using the QIAprep Spin Miniprep Kit (Qiagen), and sequenced with the aid of a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Database searches and similarity analyses of cDNA and gDNA nt sequences were performed with the BLASTN and BLASTX programs [39, 40] against public nt and protein databases, and a *P. yezoensis* genome database (Genome Assembly Ver. 1 Scaffold, http://nrifs.fra.affrc.go.jp/ResearchCenter/5_AG/genomes/nori/index.html "Accessed 2 Sep 2015") [41].

Multiple alignment and neighbor-joining-based phylogenetic tree construction for the deduced AA sequences of the *PyDUR3* proteins and the selected *DUR3* and *SSS* superfamily proteins were performed using the MEGA6 software [42] incorporating the ClustalW program [43]. The alignment parameters are as follows: gap opening penalty, 10.0; gap extension penalty, 0.2; and percent identity delay, 30; protein weight matrix, Gonnet. Bootstrap resampling analysis from 1,000 replicates was used to evaluate internal branches. Local bootstrap probabilities are indicated for branches with over 50% support. The DDBJ/EMBL/GenBank accession numbers of the deduced AA sequences analyzed are listed in Table 2. Prediction of TDs of the deduced AA sequence was carried out with the TMHMM Server v2.0 (The Center for Biological Sequence Analysis, Lyngby, Denmark: <http://www.cbs.dtu.dk/services/TMHMM/>). In addition, protein motif and transit peptide searches in the deduced AA sequence were carried out with the MOTIF (Kyoto University Bioinformatics Center: <http://www.genome.jp/tools/motif/>) and iPSORT (Human Genome Center, The

University of Tokyo: <http://ipsort.hgc.jp/>) [44] programs.

Expression analysis

Five micrograms of total RNA treated with DNase were used for ss cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative-PCR (qPCR) with the ss cDNA as the template was performed using a 7300 Real-Time PCR System (Applied Biosystems) and FastStart Universal Probe Master (ROX) (Roche Diagnostics, Basel, Schweiz) according to the supplier's instructions. Three sets of forward and reverse primers and FAM-labeled TaqMan MGB probes were designed to specifically amplify 57, 71, and 55 bp fragments for *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* mRNAs, respectively. The forward and reverse primers and the TaqMan MGB probe sets were *PyDUR3.3*-QPF/QPR/QPP for *PyDUR3.3* mRNA, *PyDUR3.2*-QPF/QPR/QPP for *PyDUR3.2* mRNA, and *PyDUR3.1*-QPF/QPR/QPP for *PyDUR3.1* mRNA (Table 1). Each standard curve of qPCR for *PyDUR3.3*, *PyDUR3.2*, and *PyDUR3.1* mRNAs was obtained from PCR amplification with diluted plasmid including the respective cDNA as the template. Normalization of the mRNA level of each *PyDUR3* gene was performed with an index of 18S rRNA detected using the TaqMan Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) including the VIC-labeled MGB probe. The data were analyzed by one-way analysis of variance (ANOVA) and Tukey's post-hoc test, with $p < 0.01$ between two experimental groups regarded as significant.

Results

Cloning and sequence analysis of *PyDUR3.3* cDNA

A *PyDUR3.3* cDNA fragment (approximately 1.7 kbp) was amplified by PCR based on 3' RACE procedure using a combination of primers, *PyDUR3.3*-EP1 and AUAP (Table 1). BLAST analysis showed that the deduced AA sequence for the cDNA fragment was homologous to *P. yezoensis* *PyDUR3.2* [19]. The poly(A) tail was not included in the cDNA fragment, because the cDNA contained an AUAP at the 3'-terminus. In order to isolate the 3' end of the *PyDUR3.3* cDNA, 3' RACE

PCR amplification was performed using the gene-specific primer PyDUR3.3-EP2 (Table 1), which was synthesized on the basis of the nt sequence of the AUAP-containing *PyDUR3.3* cDNA, and AUAP. A *PyDUR3.3* cDNA fragment (approximately 0.7 kbp), including a poly(A) tail and overlapping with the AUAP-containing *PyDUR3.3* cDNA, was successfully amplified. Next, by the PCR amplification for 5' RACE, the cDNA fragments (approximately 0.6 kbp) were cloned. Finally, based on three overlapping clones obtained by the 3' and 5' RACE procedures, the nt sequence of 2,416 bp, which includes the entire coding region of 2,040 bp, was determined (DDBJ/EMBL/GenBank accession number AB931115) (Table 2, Fig. 1).

The *PyDUR3.3* cDNA had an open reading frame (ORF) encoding a polypeptide of 679-AA residues (Fig. 1) with a molecular mass of 71.4 kDa. An AA sequence alignment of *PyDUR3.3* with *P. yezoensis* *PyDUR3.1* and *PyDUR3.2* and with some published algal, higher plant, and yeast DUR3 proteins showed that the *PyDUR3.3* is more similar to *PyDUR3.2* (80.7% identity) than to other DUR3 proteins including *PyDUR3.1* (36.8–56.7% identities) (Fig. 2). Hydrophobicity plots calculated with the TMHMM Server v2.0 showed 15 TDs for *PyDUR3.3*, and the positions of 1st and 2nd TDs from the N-terminus of the *PyDUR3.3* differed from those of *PyDUR3.1* and *PyDUR3.2*. The DUR3 proteins belong to the SSS superfamily which catalyzes the uptake of a wide variety of solutes into cells of prokaryotes and eukaryotes [16, 18, 22, 24]. Protein sequence analysis by MOTIF and iPSORT programs indicated that *PyDUR3.3* included a SSS family-specific domain (84th–494th AAs) as well as did *PyDUR3.1* (92nd–499th AAs) and *PyDUR3.2* (86th–496th AAs), and that no mitochondrial or chloroplast transit peptides were included in *PyDUR3.3/3.2/3.1*. To investigate the relationships of the predicted *PyDUR3.3* protein to its counterparts, including different family members of the SSS superfamily, from other algae, land plants, and bacteria, a neighbor-joining-based phylogenetic tree was constructed on the basis of multiple alignment of AA sequences from the selected SSS superfamily proteins (Fig. 3). Phylogenetic analysis indicated that *PyDUR3.3* fell into the red algal DUR3 cluster, and that *PyDUR3.3* was more similar to DUR3 proteins, including *PyDUR3.1* and *PyDUR3.2*, from red algae (56.7–80.7% identities) than to those from other algae (37.0–54.0% identities), land plants (49.2–50.4% identities), and yeasts (30.3–37.6% identities).

Determination of *PyDUR3* gene structures

Genomic copies of three *PyDUR3* genes were amplified by LA-PCR using three primer sets (Table 1), designed on the basis on the corresponding *PyDUR3* cDNA nt sequences, with gDNA as the template. Partial gDNA fragments, 2,755 bp for *PyDUR3.3* (DDBJ/EMBL/GenBank accession number AB933540), 2,573 bp for *PyDUR3.2* (AB933541), and 4,201 bp for *PyDUR3.1* (AB933542), were isolated. Each gDNA fragment included 5' and 3'-untranslated regions (UTR) of exons encoding translational start and stop codons, respectively. In *PyDUR3.3/3.2*, three exons (E1–3) were assigned to the coding region by comparison with the corresponding cDNA nt sequences (Fig. 4). Lengths of the ORF coding regions in E1–3 were 900, 22, and 1,118 bp, respectively, for *PyDUR3.3*, and 906, 22, and 1,115 bp, respectively, for *PyDUR3.2* (Table 3). In contrast, the coding region of *PyDUR3.1* was split into eight exons (E1–8) (Fig. 4) in which lengths of the ORF coding regions were 252, 100, 70, 457, 157, 5, 199, and 983 bp, respectively (Table 4). Introns were located at codons specifying the 300th/301st (I1) and 308th (I2) AAs for *PyDUR3.3*, 302nd/303rd (I1) and 310th (I2) AAs for *PyDUR3.2*, and 84th/85th (I1), 118th (I2), 141st (I3), 293rd/294th (I4), 346th (I5), 347th/348th (I6), and 414th (I7) AAs for *PyDUR3.1*. The consensus GT donor and AG acceptor sequences at the 5'- and 3'-termini of the introns [45, 46], respectively, were found in all introns except for the I5 in *PyDUR3.1*. BLAST analysis identified discrete contigs corresponding to *PyDUR3.3* (contig_34496), *PyDUR3.2* (contigs_30224/23844), and *PyDUR3.1* (contig_8966) (Fig. 4), suggesting that *PyDUR3.3/3.2/3.1* genes were encoded separately in the nuclear genome of *P. yezoensis*. The nt sequences of the *PyDUR3.3/3.2* gDNA fragments were identical to those of the corresponding contigs, whereas slight differences in the nt sequences of introns I1 and I2 (90.7–99.5% identities) were found between *PyDUR3.1* gDNA fragment and contig_8966.

Expression of *PyDUR3* genes in *P. yezoensis* thalli and conchocelis

The mRNA levels for *PyDUR3.3/3.2/3.1* in *P. yezoensis* thalli and conchocelis were examined by qPCR using three combinations of gene-specific primers and

TaqMan MGB probes (Table 1, Fig. 4). For the expression assay, *P. yezoensis* thalli and conchocelis maintained in 1/5 PES media were transferred to 0 PES (natural seawater) media and cultivated for 72 h (thalli) or 168 h (conchocelis) until discoloration was observed. Although the DIN of the initial 0 PES media corresponding to 0 h cultivation (Fig. 5, sample N) was approximately 258 µg/L, the DIN decreased to approximately 10 µg/L after 24 h cultivation and remained at that level until end of the culture period, indicating that thalli and conchocelis cultivated for 72 h and 168 h (Fig. 5, sample D), respectively, were exposed to a N-deficient condition and almost the same physiological condition. In order to compare *PyDUR3* gene expression profiles between the heteromorphic generations, the mRNA level of the *PyDUR3* genes was normalized using *18S rRNA*, which is a reliable internal standard in plants under various growth stages, cultivars, and stressful conditions [47], as a reference gene. *PyDUR3.3* mRNA were present at lower levels in thalli, whereas the mRNA levels were extremely high in the conchocelis (approximately 340–480-fold that of the initial thalli), regardless of the nutritional conditions. In contrast to *PyDUR3.3* expression, remarkable differences were observed in *PyDUR3.2* expression. The relative mRNA level of *PyDUR3.2* was approximately 27.6 times higher in the thalli cultivated for 72 h than in the initial thalli, and was extremely low (approximately 0.2-fold that of the initial thalli) in the conchocelis cultivated for 0 h and 168 h. On the other hand, the relative mRNA levels of *PyDUR3.1* in the thalli and conchocelis cultivated under the N-deficient condition changed approximately 3.0 and 1.5-fold compared to those in the initial thalli and conchocelis, respectively.

Discussion

In this study, a new gene, *PyDUR3.3*, which is homologous to plant and yeast high-affinity urea transporter genes, was isolated from *P. yezoensis* by RACE-PCR. The *PyDUR3.3* cDNA encoded a putative protein of 679 AAs (Fig. 1), and the primary structure of the *PyDUR3.3* differed from those of the *PyDUR3.1* and *PyDUR3.2* [19]. Structurally, the *DUR3* proteins belong to the SSS superfamily, which includes more than one hundred members of prokaryotic and eukaryotic origin and is divided into 11 subfamilies [22]. In addition, proteins of the SSS superfamily contain 11 to 15 putative TDs in an α -helical conformation, some of

which has been described to mediate uptake and transport of various solutes (e.g., AAs, inositols, nucleosides, sugars, and urea) across cytoplasmic membranes [16, 18, 24, 48]. Among all SSS proteins, the DUR3 members are closest to the bacterial sodium/pantothenate symporters (PanF) and sodium/proline symporters (PutP). Sequence analysis of the deduced AA sequence of PyDUR3.3 showed that PyDUR3.3 contained 15 putative TDs found in most of the DUR3 proteins from photosynthetic eukaryotes and yeasts, although the AA sequences were quite varied in length (653–818 AAs) (Table 2) and identity (36.8–80.7%) (Fig. 2) among the DUR3 protein family. Phylogenetic analysis indicated that the DUR3 proteins from photosynthetic eukaryotes and yeasts roughly fell into the corresponding clusters distinguished with each division, and that the DUR3 proteins from Rhodophyta (56.0–80.7% identities among their AA sequences) were more similar to those from Heterokontophyta (45.5–54.0% identities) and Chlorophyta (40.6–50.5% identities) than those from Haptophyta (29.1–43.7% identities) and Ascomycota (27.5–37.6% identities) (Fig. 3). In the Rhodophyta cluster, the PyDUR3.3 and PyDUR3.2 were phylogenetically closely related to each other (80.7% identity), whereas the PyDUR3.1 was clearly distinguished from PyDUR3.3/3.2 and more similar to a *Chondrus crispus* CcDUR3.1 (60.6% identity) than the PyDUR3.3/3.2 (56.0–56.7% identities). These results suggest that *PyDUR3.3/3.2* differs considerably from *PyDUR3.1* in ancestry and evolution.

To date, the *DUR3* gene in land plants has been investigated and found to be a single copy gene on the basis of EST and gDNA database searches. In addition, *A. thaliana* AtDUR3, *Oryza sativa* OsDUR3, and *Zea mays* ZmDUR3 proteins have been functionally characterized to mediate urea/proton cotransport at low external urea concentrations by functional complementation analysis of these plant *DUR3* genes in a yeast *DUR3*-knockout mutant, as well as physiological analysis of these plant *DUR3*-insertion transgenic lines [16, 24, 27–29]. On the other hand, two *DUR3* homologues have been identified in algae such as *C. crispus* (Rhodophyta), *Emiliania huxleyi* (Haptophyta), and *Chlamydomonas reinhardtii* (Chlorophyta) with the completion of whole genome sequencing [30, 31, 49], although the functional characteristics of their products are still unclear. In *P. yezoensis*, the *PyDUR3* genes are also encoded by multigene families [19], and the exon-intron structures of *PyDUR3.3/3.2* were closely related to each other, which clearly differed from that of *PyDUR3.1* (Fig. 4). In the yeasts *S. cerevisiae* and

Schizosaccharomyces pombe, multicellular red alga *C. crispus*, and unicellular green alga *Ostreococcus tauri*, an ancient member of the terrestrial green lineage [50], no introns were found in ORFs of the *DUR3* genes, whereas ORFs of the *DUR3* genes in other algae and land plants studied is interrupted by two (e.g., *OsDUR3*) to nine (e.g., *EsDUR3* and *AtDUR3*) introns with taxon-nonspecific locations (Table 3). Interestingly, in the unicellular algae *E. huxleyi* and *C. reinhardtii*, each having two *DUR3* genes, the exon-intron structure of one *DUR3* gene (*EhDUR3.1* and *CrDUR3B*) is more complicated than that of the other *DUR3* gene (*EhDUR3.2* and *CrDUR3A*). In *P. yezoensis* *PyDUR3* genes, the same structural feature was observed between *PyDUR3.3/3.2* and *PyDUR3.1* (Table 3, Fig. 4), strongly supporting that *PyDUR3.3* might be a paralogue of *PyDUR3.2*. Amino acid sequence and phylogenetic analyses also support this conclusion (Figs. 2 and 3).

Generally, *Pyropia* thalli can utilize inorganic nitrogen and organic solutes like AAs and urea in growth and development [9, 12–15]. In addition, N-deficiency in seawater leads *Pyropia* thalli and conchocelis to discoloration with degradation of phycobiliproteins, the predominant photosynthetic pigments in red algae [9–11]. Therefore, multiple *PyDUR3* genes in *P. yezoensis* may be responsible for uptake of a large variety of solutes in seawater and for intracellular transport of endogenous urea liberated via degradation of phycobiliproteins, as urea metabolism in plant cells [18]. In land plants, a *DUR3* protein is specific for urea and acts as a major high-affinity urea transporter not only for direct uptake of exogenous urea in roots but also for retranslocation or recycling of endogenous urea liberated via degradation of storage proteins in germinating seeds and mature leaves [24–27, 29]. Land plant *DUR3* gene expression is up-regulated in roots under N-deficiency, in seeds during early germination, and in mature leaves during senescence. In contrast, yeast *S. cerevisiae* *ScDUR3* has been reported to mediate uptake of extracellular urea and polyamines [51]. Compared to the information for land plant and yeast *DUR3* genes, far less is known about molecular characteristics of algal *DUR3* genes. Therefore, changes in mRNA levels of the three *PyDUR3* homologs in thalli and conchocelis were examined under different nutrient conditions (Fig. 5), and the results suggested that expression of the *PyDUR3.3* gene is constitutive and specific to the conchocelis, whereas *PyDUR3.2* gene expression is specific to the thalli and significantly affected by exposure to nitrate starvation, and that *PyDUR3.1* gene expression is positively regulated by nitrate starvation in

both thalli and conchocelis.

The *Pyropia* life cycle alternates between two growth phases, a leafy thallus as the haploid gametophyte and a filamentous conchocelis as the diploid sporophyte [1]. The *Pyropia* thalli appear in nature as free-living organisms during the winter with low temperature and short day length, and the conchocelis burrows into calcium carbonate (CaCO_3) substrates such as oyster shells and grows during the summer with high temperature and long day length. Shellfish excrete urine including nitrogenous compounds such as $\text{NH}_4\text{-N}$, urea-N, and AA-N [52]. In addition, biogenic polyamines accelerate the formation of CaCO_3 in seawater [53], suggesting that a similar phenomenon may be involved in the calcification process in shellfish. *PyDUR3.3* was constitutively expressed in the conchocelis (Fig. 5), suggesting a possibility that *PyDUR3.3* plays an especially important role in the uptake of nitrogenous compounds excreted from shellfish. In plants and algae, polyamines are ubiquitous cellular components and play important roles in fundamental cellular processes, including adaptive/defensive responses to various environmental stresses [54–58]. Expression of *PyDUR3.2* and *PyDUR3.1* genes were induced by N-deficiency in both generations (Fig. 5), suggesting that these *PyDUR3* proteins play especially important roles in the uptake of nitrogenous compounds in N-deficient conditions, as well as in transport of endogenous urea produced from degradation of proteins in the discoloration process and polyamines involved in stress responses.

In this study, three *PyDUR3* genes could be characterized on the basis of the sequence similarities, genomic organization, and expression profiles in heteromorphic generations. Experimental results suggest that *PyDUR3.3* and *PyDUR3.2* are paralogues specifically differentiated in function and life history phase, which clearly differed from *PyDUR3.1* in ancestry and evolution. However, transport mechanisms including substrate specificity and subcellular localization of each *PyDUR3* remain unclear. Plant *DUR3* genes have been characterized by functional complementation analysis using a yeast *DUR3*-knockout mutant, electrophysiological and radiotracer analyses of *DUR3*-injection *Xenopus* oocytes, and physiological analysis of *DUR3*-insertion transgenic lines [16, 24, 27–29]. Further experiments for the functional characterization of these *PyDUR3* genes are currently being carried out using the tools mentioned above.

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Figure legends

Fig. 1 Nucleotide sequence of the *P. yezoensis* PyDUR3.3 cDNA. The deduced AA sequence is shown below the nt sequence. Numbers in the margin represent nt and AA residues from the 5'-end and N-terminus, respectively. The deduced AA sequence is shown in *single letter code*. The stop codon (TAG) is marked by an *asterisk*. *Bold small letters* in the AA sequence indicate the putative TDs. *Underlined* and *dashed underlined* nt sequences correspond to primers for RACE-PCR (PyDUR3.3-EP1/EP2 and PyDUR3.3-SP1/SP2/SP3) and LA-PCR (PyDUR3.3-GF/GR), and for qPCR (PyDUR3.3-QPF/QPP/QPR), respectively, listed in Table 1. *Arrowheads* with the primers indicate nt sequence direction from 5' to 3'-ends. The *double underlined* nt sequence shows high similarity to the AUAP primer used for 3' RACE. Refer to Table 2 for the DDBJ/EMBL/GenBank accession number of the PyDUR3.3 cDNA sequence.

Fig. 2 Comparison of AA sequences of DUR3 proteins from *P. yezoensis* with those of other algae, higher plants, and yeasts. The deduced AA sequence of *P. yezoensis* PyDUR3.3 (identified in this study) is shown on the upper line in *single letter code*. The AA sequences of *P. yezoensis* PyDUR3.2 and PyDUR3.1 [19], *E. siliculosus* EsDUR3 [59], *C. reinhardtii* CrDUR3A [30], *A. thaliana* AtDUR3 [24], and *S. cerevisiae* ScDUR3 [23] are shown below. Numbers in the margin represent AA residues from the N-terminus. Identical and gapped AAs are shown by *periods* and *dashes*, respectively. Below the AA sequences, *asterisks* indicate AA residues conserved in all sequences, and *double/single dots* denote highly/moderately conservative replacements. *Underlined bold small letters* in each AA sequence indicate the putative TDs. After each AA sequence, percentage AA sequence identity relative to PyDUR3.3 and numbers of TDs are given in *parentheses*. Refer to Table 2 for the DDBJ/EMBL/GenBank accession numbers of the DUR3 protein sequences.

Fig. 3 Neighbor-joining-based phylogenetic tree of DUR3 and SSS superfamily proteins obtained using the ClustalW and MEGA6 programs [42, 43]. Boxes indicate *P. yezoensis* PyDUR3 proteins. The bootstrap values with 1,000 replicates over 50% are indicated at the nodes of the tree. Refer to Table 2 for the

DDBJ/EMBL/GenBank accession numbers of the DUR3 and SSS superfamily protein sequences analyzed.

Fig. 4 Schematic representation of gDNA clones encoding the *PyDUR3* genes. Horizontal **black** bars and boxes represent introns and exons, respectively. Shaded boxes and open/light-shaded boxes represent UTRs of exons and *PyDUR3* coding exons, respectively. In boxes, light-shaded regions indicate TD coding exons. The partial gDNA fragments, 2,755 bp for *PyDUR3.3* (DDBJ/EMBL/GenBank accession number AB933540), 2,573 bp for *PyDUR3.2* (AB933541), and 4,201 bp for *PyDUR3.1* (AB933542), were isolated. Horizontal gray bars represent scaffolds in the *P. yezeensis* genome database [41] corresponding to the *PyDUR3* gDNA fragments. Arrows and arrowheads indicate the *PyDUR3* gene-specific primers for LA-PCR (*PyDUR3.3*-GF/GR, *PyDUR3.2*-GF/GR, and *PyDUR3.1*-GF/GR) and qPCR (*PyDUR3.3*-QPF/QPP/QPR, *PyDUR3.2*-QPF/QPP/QPR, and *PyDUR3.1*-QPF/QPP/QPR), respectively, listed in Table 1.

Fig. 5 Expression of the *PyDUR3* genes in *P. yezeensis* thalli and conchocelis. *Pyropia* thalli and conchocelis maintained in 1/5 PES media were transferred to seawater without PES enrichment (DIN = 258 µg/L) (0 h, N) and cultivated for 72 h or 168 h (D) in the case of the thalli or conchocelis, respectively. The relative mRNA levels of each *PyDUR3* gene were normalized with the amount of 18S rRNA and given as relative fold abundance compared to the initial thallus N sample. Error bars indicate the standard deviation of triplicate experiments ($n = 3$). Different letters indicate significant differences ($p < 0.01$).

744 **Table 1** Nucleotide sequences of primers for PCR amplifications

Primer name	Sequence
3' RACE for PyDUR3.3	
PyDUR3.3-EP1	5'-dTTTGACAACAAGCCCGTCCTGGACAAG-3'
PyDUR3.3-EP2	5'-dCAAGCCAGACAACCTGTGACTGGTC-3'
AUAP	5'-dGGCCACGCGTCGACTAGTAC-3'
5' RACE for PyDUR3.3	
PyDUR3.3-SP1	5'-dATGACAGCCGTATTGAAGTAG-3'
PyDUR3.3-SP2	5'-dTGCCAGGTTGGTACTGATACC-3'
PyDUR3.3-SP3	5'-dAACAATAACGTTGGTAGCCAGCGC-3'
LA-PCR	
PyDUR3.3-GF	5'-dATCCCAAGTCTCTCTTTCTGGTCGTAC-3'
PyDUR3.3-GR	5'-dCAACCTGCTATAGTGAACCAGCTTACC-3'
PyDUR3.2-GF	5'-dGTTCCCACCATGGCGGACTTTGTCAAC-3'
PyDUR3.2-GR	5'-dAGGCCGCGCAAAAGACCGTCAGTCTAT-3'
PyDUR3.1-GF	5'-dCGGGAGGTGTTGGCGCAAAAATGGCGA-3'
PyDUR3.1-GR	5'-dCACAGCTCTACACCTACTCGCAACACG-3'
Quantitative-PCR	
PyDUR3.3-QPF	5'-dCTGCCGACGGCGAGTCTA-3'
PyDUR3.3-QPR	5'-dGCCACCTGTTTGCGTTGAT-3'
PyDUR3.3-QPP	5'-dACGAGTAGCCGTTGCG-3'
PyDUR3.2-QPF	5'-dCGAGAGCGACGGCATCTC-3'
PyDUR3.2-QPR	5'-dATCCGAGCCGACCCTCTT-3'
PyDUR3.2-QPP	5'-dCCGAGGTGGACGAGTAG-3'
PyDUR3.1-QPF	5'-dTGGAACCACCAGAGCCTTCT-3'
PyDUR3.1-QPR	5'-dCGCGCAACGGTGATCA-3'
PyDUR3.1-QPP	5'-dACCGCACATCGTTCT-3'

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Table 2 DDBJ/EMBL/GenBank accession numbers of DUR3 and SSS protein sequences

Species	Proteins	Abbreviations	AAs	TDs ^{a)}	Accession No.
Urea active transporter protein					
Rhodophyta					
<i>Chondrus crispus</i>	DUR3.1	CcDUR3.1	675	15	HG001812
	DUR3.2	CcDUR3.2	669	15	HG001512
<i>Pyropia yezoensis</i>	DUR3.1	PyDUR3.1	740	15	AB359179
	DUR3.2	PyDUR3.2	680	15	AB359180
	DUR3.3	PyDUR3.3	679	15	AB931115
Heterokontophyta					
<i>Ectocarpus siliculosus</i>	DUR3	EsDUR3	668	15	FN647726
<i>Phaeodactylum tricornutum</i>	DUR3	PtDUR3	704	15	CM000612
Haptophyta					
<i>Emiliania huxleyi</i>	DUR3	EhDUR3.1	756	14	KB864035
	DUR3	EhDUR3.2	818	13	KB868914
Chlorophyta					
<i>Ostreococcus tauri</i>	DUR3	OtDUR3	710	15	Q00V32
<i>Chlamydomonas reinhardtii</i>	DUR3A	CrDUR3A	653	15	DS496184
	DUR3B	CrDUR3B	721	15	DS496184
<i>Physcomitrella patens</i>	DUR3	PpDUR3	713	15	DS545146
<i>Oryza sativa</i>	DUR3	OsDUR3	721	15	AY463691
<i>Zea mays</i>	DUR3	ZmDUR3	731	15	KM271989
<i>Arabidopsis thaliana</i>	DUR3	AtDUR3	694	14	AB018113
<i>Vitis vinifera</i>	DUR3	VvDUR3	710	15	XM_002263007
Ascomycota					
<i>Saccharomyces cerevisiae</i>	DUR3	ScDUR3	735	15	NC_001140
<i>Schizosaccharomyces pombe</i>	DUR3	SpDUR3.1	664	15	NC_003423
	DUR3	SpDUR3.2	661	15	NC_003424
	DUR3	SpDUR3.3	673	14	NC_003423
<i>Neurospora crassa</i>	DUR3	NcDUR3	704	15	CM002236
Bacterial proline symporter					
<i>Bacillus subtilis</i>	PutP	BsPutP	473	12	AL009126
<i>Corynebacterium glutamicum</i>	PutP	CgPutP	524	13	Y09163
<i>Helicobacter pylori</i>	PutP	HpPutP	496	13	AE000511
<i>Neisseria meningitidis</i>	PutP	NmPutP	508	13	AE002098
<i>Vibrio vulnificus</i>	PutP	VvPutP	497	12	AF454004
Bacterial pantothenate symporter					
<i>Bacillus cereus</i>	PanF	BcPanF	479	13	AAEK01000037
<i>Escherichia coli</i>	PanF	EcPanF	483	13	CP001855
<i>Fusobacterium nucleatum</i>	PanF	FnPanF	484	12	AE009951
<i>Salmonella enterica</i>	PanF	SePanF	483	13	CP000857
<i>Shigella flexneri</i>	PanF	SfPanF	483	13	CP000266
<i>Yersinia pestis</i>	PanF	YpPanF	486	13	AE009952
Bacterial other sodium solute symporter					
<i>Pseudomonas aeruginosa</i>	gpuP	PaGpuP	461	13	AE004091
<i>Ralstonia solanacearum</i>	SSS	RsSSS	479	13	AL646052
<i>Vibrio parahaemolyticus</i>	SGLT	VpSGLT	543	14	AF255301

^{a)} TDs in the deduced AA sequences were predicted by TMHMM version 2.0.

751 **Table 3** Gene structure of *DUR3* genes

<i>DUR3</i> genes	Lengths (bp) of ORF coding regions in exons									
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Rhodophyta										
<i>CcDUR3.1</i>	2028									
<i>CcDUR3.2</i>	2010									
<i>PyDUR3.1</i>	252	100	70	457	157	5	199	983		
<i>PyDUR3.2</i>	906	22	1115							
<i>PyDUR3.3</i>	900	22	1118							
Heterokontophyta										
<i>EsDUR3</i>	33	49	42	56	524	294	442	124	427	16
<i>PtDUR3</i>	221	64	7	55	675	1093				
Haptophyta										
<i>EhDUR3.1</i>	105	255	443	583	214	195	176	300		
<i>EhDUR3.2</i>	96	219	64	1404	674					
Chlorophyta										
<i>OtDUR3</i>	2133									
<i>CrDUR3A</i>	116	176	766	484	420					
<i>CrDUR3B</i>	67	27	70	176	776	320	154	105	114	357
<i>PpDUR3</i>	177	108	400	260	239	958				
<i>OsDUR3</i>	183	771	1212							
<i>ZmDUR3</i>	192	108	663	1233						
<i>AtDUR3</i>	168	108	96	83	221	263	239	431	204	272
<i>VvDUR3</i>	174	108	96	83	221	263	239	650	299	
Ascomycota										
<i>ScDUR3</i>	2208									
<i>SpDUR3.1</i>	1995									
<i>SpDUR3.2</i>	1986									
<i>SpDUR3.3</i>	2022									
<i>NcDUR3</i>	106	1664	345							

752

PyDUR3.3-GF>

ATCCCAAGTCTCTCTTTCTGGTCGTACCTCTTCCCGTTCTCGGGTTTCCGAGTTGCCGGCGTCATGGCGACTCCGGCC	78
GTGGTCAATCCTGGGATTGCCGCGTGGGGCTGCGCAGCAACGGTAGTTCGGAACCTTCTTCCTCGACCCTCAGTATGAG	156
<i>v v n p g i a a w g c a a t v v r n f f l D P Q Y E</i>	31
PyDUR3.3-EP1>	
TGTCGGGAAAAGCTTCTTTGACAACAAGCCCGTCTCGGACAAGTGGGTCGGCTATGTTATTGTGCTCGCATTTGGCGTT	234
C R E S F F D N K P V L D K W <i>v g y v i v l a f g v</i>	57
GCTTTTGGCATTGCCACCGTTGGCATCGTGCTGATTGAGCAGCGGGTGCTCGGGCGCAAGATGGACTCTGAGTTCTTC	312
<i>a f g i a t v g i v l i E Q R V L G R K M D S E F F</i>	83
AACACGGCCGGGCGGAGTGTCAGACGGGCCTCACGGCCTCGGTGATTGTGTCGCAAGTGACGTGGGCCGCGACGCTG	390
N T A G R S V K T G L T A S V I V S Q W T W A A T L	109
CTGCAGTCATCCAATGTGGCGTTCAAGTATGGGGTGCTGGGCCGTTTTGGTACGCGTCCGGCGCAACGCTGCAAAATC	468
L Q S S N V A F K Y G V S <i>g p f w y a s g a t l q i</i>	135
ATTGTCTTCTCGCTGCTGGCGGTGTCAGGTCAAGCGGCGGGCCCAACCGCCCAACCTTCCTTGAGATCATCCAAGCG	546
<i>i v f s l l a v q v K R R A P T A H T F L E I I Q A</i>	161
<PyDUR3.3-SP3	
CGGTGGGGGACGGTCGCGCACGCCGTATTTCTCTTTTTCGCTGGCTACCAACGTTATTGTTACCTCTATGCTCATC	624
R W G T <i>v a h a v f l f f a l a t n v i v t s m l i</i>	187
<PyDUR3.3-SP2	
CTTGGCGGCAGCGCGGTGGTCAGCGCGCTGACGGGTATCAGTACCAACCTGGCATCCTTCCTCATTCCCTGGGAGTC	702
<i>l g g s a v v s a l t g i s t n l a s f l i p l g v</i>	213
<PyDUR3.3-SP1	
ATTCTCTACACCCTTGCGGGGGGACTCAAAGCGACTTTTGTGGCGTCTACTTCAATACGGCTGTCACTCTCATTGCT	780
<i>i l y t l a g g l k a t f v a s y f n t a v i l i a</i>	239
CTTTGCATCTTTGTCTTCCAGGTCTACGTGACTGACGCCACGCTGGGGTCCCGTACTAGTCTACGACCGGCTGCAA	858
<i>l c i f v f q v y v T D A T L G S P S A V Y D R L Q</i>	265
GAGTCGGTGTCTGTTGAACCAAGTAGTGACAACCGCGGTGGCAGCTACCTCACCATGTTTCAGCAAGAACGGGCTTCTG	936
E S V S F E P V V D N R G G S Y L T M F S K N <i>g l l</i>	291
TTTGGGCTGAGCAACATTTGCGGCAATTTTGGCACCGTGTTTGTGGACCAGAGCTACTGGCAGTCTGCAATCGCGGCG	1014
<i>f g l s n i c g n f g t v f v D Q S Y W Q S A I A A</i>	317
ACTCCACAGGCCGCTTGGAAGGGCTACATCCTGGGTGGTCTGTCTGCTGTTTTCATCCCTTTTACGCTGGCCACGTCG	1092
T P Q A A W K G <i>y i l g g l s w f s i p f t l a t s</i>	343
TTGGGCTGGCTGGTCTCGCCCTGTGCTGCTATCACTATCGATGAGTCCAACAGTGGGCTAGTCCCGCCTGCCGTG	1170
<i>l g l a g l a l s l p i t i d e s n s g l v p p a v</i>	369
GCCACCCATCTCATGGGCAAGGGTGGCTCAGTGCTCATCTCATTATGCTCTTTATGGCTGTGACGTCTACCGGCGCG	1248
<i>a t h l m g k g g s v l i i m l f m a v t s t g a</i>	395
GCCGAGCAGATCGCTGTTTCTTCTCGCTCGTCTGCTTACGACATTTACGTGCCCATCCGGCGGCATATGGGCCATAACCT	1326
A E Q I A V S S L V A Y D I Y V P I R R H M G H N P	421
ACTGGCAAGGAGATCATCCTTGTCTCCCGCATTGCCATTGTGGCGTTTGGCTTGTGATGGGGGTTCTGGGCATCGCG	1404
T G K <i>e i i l v s r i a i v a f g l l m g v l g i a</i>	447
CTCAACGCGATCGGCGTCAGCCTTGGGTGGTCTACCTTGGCATGGGGTTCATATTGGGTCCGCGGTGCCCCCTGTT	1482
L N A I G V S L G <i>w v y l g m g v i i g s a v a p v</i>	473
GCCATGTCCATCAGCTGGGCAAAGTGCTCCGGGGCGGGGGCTATATCTGGAGCCGTCGGTGGGCTGGCCCTCGCCATT	1560
<i>a m s i s w A K C S G A g a i s g a v g g l a l a i</i>	499
GCCTCGTGGCTCGGGTACGCCTCTACCTTTGATGGGGGCGTCAACATTGCAAACACGGGCCTTGACCAGGTCTGGTC	1638
<i>a s w l g y a s t F D G G V N I A N T G L D Q v m v</i>	525
PyDUR3.3-EP2>	
GTTGGCAACTTGGTGGCCATCCTCAGCTCCGCCGTATCTGTGCAAGTTGTGTCTTTCATCAAGCCAGACAACCTGTGAC	1716
<i>v g n l v a i l s s a v i c a v v s f i K P D N C D</i>	551
TGGTCGGCGACCAAGGCGATTGCGCTCGTCGACGAGGATGTCAACGCAGAGCTCTCGCCCCGAGGACGAGGCGGAGATT	1794
W S A T K A I A L V D E D V N A E L S P E D E A E I	577
GATCGCGCCATGAAGATGATCAGCGCGTGGGGCATTGGCCTTGCCTGCTGGTCTGCTCGCGTGGCCGCTGCTGGCG	1872
D R A M K M I S A <i>w g i g l a l v l v v a w p l l a</i>	603
CTGCCTGCGGGCGTTTTCTCAAAGGGCTACTTCACGTTCTGGGTGTTCTCTCGATCATCTGGGGTATCATGGCCACG	1950
<i>l p a g v f S K G y f t f w v v l s i i w g i m a t</i>	629
GGCGCATGGTGTCTCCCGTGTTGGGAGTCGCGGTCGTCATCTCGGGGTTATTACTCTGGGTAAAGATCGTACCG	2028
<i>g a m v l l P V W E S R S I L G V I T L G K I V P</i>	655
PyDUR3.3-QPF>	
PyDUR3.3-QPP>	
ACGCCAGAGCCCAAGAGCCTTGAGGACTCGTTGGAGCTGGCAGAGTCTGCCGACGGCGAGTCTACGAACGAGTAGCCG	2106
T P E P K S L E D S L E L A E S A D G E S T N E *	679
<PyDUR3.3-QPR	
TTGCGCATCAACGCAACAGGTGGCAGACTTCGCGTGCGAAGTCCGCGTCCCATGCAGTTTTTATTGATCGCCTCTGA	2184
TCGCTGCTTGTCTTAAGTCCCGGTGGGAGTGTTGGGCCGACACTCCCTGCCGCTGCCCTTTTCGCTAGCATACGG	2262
GAGGAGTGCTGTGCGTACTGTCAACGCTTCTGTCGGTGCGACGTTTCTTGTACGTTGTCTCATGTTGTCTCTCTTG	2340
<PyDUR3.3-GR	
GCGGGGTAAGCTGGTTCACTATAGCAGTTGTTGTCTAGTGGGTTATGTCAACCGTCACAAAAAAAAAAAAAAAAAAAA	2416

PyDUR3.3	MAT	<u>pavvnpqiaawgcaat-vvrnffl</u>	DPQYECRESFFDNKP	---	VLDK	<u>wgvvivlafgvafigiatvgivli</u>	EORVLGRKMDSEFFNTAGRSVKTGLTASVIVSQ	102
PyDUR3.2	MAT	<u>DF...SVE...TTSP...T...</u>	A.OA...GLADG	SAQ	Sra	<u>a...is...l...l...R...I...N...P...</u>	...	104
PyDUR3.1	MAT	<u>SPA.IPGFNLP</u>	SHE...DSEAPW...	ELDL...K.N...	GDVSNPTH	<u>Ai.t.fvivql.s...f...ll...AL...YLFSTV...T...Y...</u>	...s...	110
ESDUR3			MTSPVED.IDQD...	GGE.P...	SEA...	<u>v...1...if.sff.tfl.wld</u>	KFN.VSIT...O...	80
CrDUR3A				MRPRSGADTA	PEGL	<u>wf.ligl.av.alvptflwadrkfg</u>	TOA...N.A...	70
AtDUR3			MATCPPDFSTKYDYG	GGCQRQS...	GGTTV...	<u>OG...avi.q.af.avf.sfl.wl</u>	K.YV.ARHT...W...	92
ScDUR3			MGEFKP	---	P	<u>POGA...a...gl.av.agmm.lttyll</u>	K.YQKEITA...E.T...	67
PyDUR3.3	WT	<u>AATLLQSSN</u>	VAFKYGVS	<u>gpfwyasgat lqiivfsl lavqv</u>	KRRAPT	<u>AHTFLEIIQARWGT vahavflffalatnvi vtsmlil</u>	GGSAVVSAL <u>tgistn lasfliplq</u>	212
PyDUR3.2								214
PyDUR3.1								220
ESDUR3								190
CrDUR3A								180
AtDUR3								202
ScDUR3								177
PyDUR3.3								302
PyDUR3.2								304
PyDUR3.1								310
ESDUR3								300
CrDUR3A								270
AtDUR3								307
ScDUR3								267
PyDUR3.3								403
PyDUR3.2								405
PyDUR3.1								411
ESDUR3								401
CrDUR3A								371
AtDUR3								408
ScDUR3								377
PyDUR3.3	LV	<u>AYDIYVPIRRHMGHNPTG</u>	<u>keiilvsriaivafql lmgvlqia</u>	LN	AGVSLG	<u>wvylgmqvliigsavapvamsisw</u>	AKCSGA <u>gaigavvgqla laiaswlgvast</u>	512
PyDUR3.2								514
PyDUR3.1								517
ESDUR3								507
CrDUR3A								478
AtDUR3								514
ScDUR3								483
PyDUR3.3	VN	<u>IAN</u>	<u>TGLDQvmvvgnlvailssavic-avvsfi</u>	KPDNCDWS	ATKAIALV	DEVDN		566
PyDUR3.2								568
PyDUR3.1								571
ESDUR3								561
CrDUR3A								533
AtDUR3								560
ScDUR3								593
PyDUR3.3								666
PyDUR3.2								668
PyDUR3.1								671
ESDUR3								663
CrDUR3A								636
AtDUR3								664
ScDUR3								703
PyDUR3.3	ELA	<u>ESADGESTNE</u>						679
PyDUR3.2	GES	<u>DGISS.VDE</u>						680
PyDUR3.1	FH	<u>ESGGGS.AGD</u>	EGGYEVGYADGFAAARA	VAAGSGMPLD	GELPDL	MGP	DGGHRLVEPPEPSHRTSF	740
ESDUR3	QAP	<u>SA</u>						668
CrDUR3A	AH	<u>KGDHME</u>	LPPAPANRA					653
AtDUR3	R	<u>RAL</u>	TMVPEA	KIYLL	ELEKTKKNDEEG			694
ScDUR3	NS	<u>NPQDLHV</u>	SQISARAHRQSSHF	GQVDEII				735





