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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 (NO_3-N), nitrite-N (NO_2-N), and ammonium-N (NH_4-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 µmol photons $m^{-2}s^{-1}$ 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 µmol photons $m^{-2}s^{-1}$ 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 µg/L (NO_3-N = 240.4 µg/L, NO_2-N = 4.5 µg/L, and NH_4-N = 12.7 µg/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. cervisiae* 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 NH_4-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. yezoensis* 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. yezoensis* 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 ($p < 0.01$).

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’-dCAAGCCAGACAACTGTGACTGGTC-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’

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					
Chondrus crispus	DUR3.1	CcDUR3.1	675	15	HG001812
	DUR3.2	CcDUR3.2	669	15	HG001512
Pyropia yezoensis	DUR3.1	PyDUR3.1	740	15	AB359179
	DUR3.2	PyDUR3.2	680	15	AB359180
	DUR3.3	PyDUR3.3	679	15	AB931115
Heterokontophyta					
Ectocarpus siliculosus	DUR3	EsDUR3	668	15	FN647726
Phaeodactylum tricornutum	DUR3	PtDUR3	704	15	CM000612
Haptophyta					
Emiliania huxleyi	DUR3	EhDUR3.1	756	14	KB864035
	DUR3	EhDUR3.2	818	13	KB868914
Chlorophyta					
Ostreococcus tauri	DUR3	OtDUR3	710	15	Q00V32
Chlamydomonas reinhardtii	DUR3A	CrDUR3A	653	15	DS496184
	DUR3B	CrDUR3B	721	15	DS496184
Physcomitrella patens	DUR3	PpDUR3	713	15	DS545146
Oryza sativa	DUR3	OsDUR3	721	15	AY463691
Zea mays	DUR3	ZmDUR3	731	15	KM271989
Arabidopsis thaliana	DUR3	AtDUR3	694	14	AB018113
Vitis vinifera	DUR3	VvDUR3	710	15	XM_002263007
Ascomycota					
Saccharomyces cerevisiae	DUR3	ScDUR3	735	15	NC_001140
Schizosaccharomyces pombe	DUR3	SpDUR3.1	664	15	NC_003423
	DUR3	SpDUR3.2	661	15	NC_003424
	DUR3	SpDUR3.3	673	14	NC_003423
Neurospora crassa	DUR3	NcDUR3	704	15	CM002236
Bacterial proline symporter					
Bacillus subtilis	PutP	BsPutP	473	12	AL009126
Corynebacterium glutamicum	PutP	CgPutP	524	13	Y09163
Helicobacter pylori	PutP	HpPutP	496	13	AE000511
Neisseria meningitidis	PutP	NmPutP	508	13	AE002098
Vibrio vulnificus	PutP	VvPutP	497	12	AF454004
Bacterial pantothenate symporter					
Bacillus cereus	PanF	BcPanF	479	13	AAEK01000037
Escherichia coli	PanF	EcPanF	483	13	CP001855
Fusobacterium nucleatum	PanF	FnPanF	484	12	AE009951
Salmonella enterica	PanF	SePanF	483	13	CP000857
Shigella flexneri	PanF	SfPanF	483	13	CP000266
Yersinia pestis	PanF	YpPanF	486	13	AE009952
Bacterial other sodium solute symporter					
Pseudomonas aeruginosa	gpuP	PaGpuP	461	13	AE004091
Ralstonia solanacearum	SSS	RsSSS	479	13	AL646052
Vibrio parahaemolyticus	SGLT	VpSGLT	543	14	AF255301

a) TDs in the deduced AA sequences were predicted by TMHHM version 2.0.

Table 3 Gene structure of *DUR3* genes

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

```
PyDUR3.3-GF>
ATCCCAAGTCTCTCTTTCTGGTCGTACCTCTTCCCGTTCTCGGGTTTCCGAGTTGCCGGCGTCATGGCGACTCCGGCC    78
                                                                M  A  T  p  a      5
GTGGTCAATCCTGGGATTGCCGCGTGGGGCTGCGCAGCAACGGTAGTTCGGAACTTCTTCCTCGACCCTCAGTATGAG   156
 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     31
               PyDUR3.3-EP1>
TGTCGGGAAAGCTTCTTTGACAACAAGCCCGTCCTGGACAAGTGGGTCGGCTATGTTATTGTGCTCGCATTTGGCGTT   234
 C  R  E  S  F  F  D  N  K  P  V  L  D  K  W  v  g  y  v  i  v  l  a  f  g  v     57
GCTTTTGGCATTGCCACCGTTGGCATCGTGCTGATTGAGCAGCGGGTGCTCGGGCGCAAGATGGACTCTGAGTTCTTC   312
 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     83
AACACGGCCGGGCGGAGTGTCAAGACGGGCCTCACGGCCTCGGTGATTGTGTCGCAGTGGACGTGGGCCGCGACGCTG   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
CTGCAGTCATCCAATGTGGCGTTCAAGTATGGGGTGTCTGGGCCGTTTTGGTACGCGTCCGGCGCAACGCTGCAAATC   468
 L  Q  S  S  N  V  A  F  K  Y  G  V  S  g  p  f  w  y  a  s  g  a  t  l  q  i    135
ATTGTCTTCTCGCTGCTGGCGGTGCAGGTCAAGCGGCGGGCCCCAACCGCCCACACCTTCCTTGAGATCATCCAAGCG   546
 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    161
                                                  <PyDUR3.3-SP3
CGGTGGGGGACGGTCGCGCACGCCGTATTTCTCTTCTTTGCGCTGGCTACCAACGTTATTGTTACCTCTATGCTCATC   624
 R  W  G  T  v  a  h  a  v  f  l  f  f  a  l  a  t  n  v  i  v  t  s  m  l  i    187
                                          <PyDUR3.3-SP2
CTTGGCGGCAGCGCGGTGGTCAGCGCGCTGACGGGTATCAGTACCAACCTGGCATCCTTCCTCATTCCCCTGGGAGTC   702
 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    213
                                                        <PyDUR3.3-SP1
ATTCTCTACACCCTTGCGGGGGGACTCAAAGCGACTTTTGTGGCGTCCTACTTCAATACGGCTGTCATTCTCATTGCT   780
 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    239
CTTTGCATCTTTGTCTTCCAGGTCTACGTGACTGACGCCACGCTGGGGTCGCCGTCCGCAGTCTACGACCGGCTGCAA   858
 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    265
GAGTCGGTGTCGTTTGAACCAGTAGTGGACAACCGCGGTGGCAGCTACCTCACCATGTTCAGCAAGAACGGGCTTCTG   936
 E  S  V  S  F  E  P  V  V  D  N  R  G  G  S  Y  L  T  M  F  S  K  N  g  l  l    291
TTTGGGCTGAGCAACATTTGCGGCAATTTTGGCACCGTGTTTGTGGACCAGAGCTACTGGCAGTCTGCAATCGCGGCG  1014
 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    317
ACTCCACAGGCCGCTTGGAAGGGCTACATCCTGGGTGGTCTGTCCTGGTTTTCCATCCCTTTTACGCTGGCCACGTCG  1092
 T  P  Q  A  A  W  K  G  y  i  l  g  g  l  s  w  f  s  i  p  f  t  l  a  t  s    343
TTGGGCCTGGCTGGTCTCGCCCTGTCGCTGCCTATCACTATCGATGAGTCCAACAGTGGGCTAGTCCCGCCTGCCGTG  1170
 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    369
GCCACCCATCTCATGGGCAAGGGTGGCTCAGTGCTCATCCTCATTATGCTCTTTATGGCTGTGACGTCTACCGGCGCG  1248
 a  t  h  l  m  g  k  g  g  s  v  l  i  l  i  m  l  f  m  a  v  T  S  T  G  A    395
GCCGAGCAGATCGCTGTTTCTTCGCTCGTCGCTTACGACATTTACGTGCCCATCCGGCGGCATATGGGCCATAACCCT  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
ACTGGCAAGGAGATCATCCTTGTCTCCCGCATTGCCATTGTGGCGTTTGGCTTGTTGATGGGGGTTCTGGGCATCGCG  1404
 T  G  K  e  i  i  l  v  s  r  i  a  i  v  a  f  g  l  l  m  g  v  l  g  i  a    447
CTCAACGCGATCGGCGTCAGCCTTGGGTGGGTCTACCTTGGCATGGGGGTCATCATTGGGTCCGCGGTCGCCCCTGTT  1482
 L  N  A  I  G  V  S  L  G  w  v  y  l  g  m  g  v  i  i  g  s  a  v  a  p  v    473
GCCATGTCCATCAGCTGGGCAAAGTGCTCCGGGGCGGGGGCTATATCTGGAGCCGTCGGTGGGCTGGCCCTCGCCATT  1560
 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    499
GCCTCGTGGCTCGGGTACGCCTCTACCTTTGATGGGGGCGTCAACATTGCAAACACGGGCCTTGACCAGGTCATGGTC  1638
 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    525
                                                           PyDUR3.3-EP2>
GTTGGCAACTTGGTGGCCATCCTCAGCTCCGCCGTCATCTGTGCAGTTGTGTCCTTCATCAAGCCAGACAACTGTGAC  1716
 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    551
TGGTCGGCGACCAAGGCGATTGCGCTCGTCGACGAGGATGTCAACGCAGAGCTCTCGCCCGAGGACGAGGCGGAGATT  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
GATCGCGCCATGAAGATGATCAGCGCGTGGGGCATTGGCCTTGCGCTGGTGCTGGTCGTCGCGTGGCCGCTGCTGGCG  1872
 D  R  A  M  K  M  I  S  A  w  g  i  g  l  a  l  v  l  v  v  a  w  p  l  l  a    603
CTGCCTGCGGGCGTTTTCTCCAAGGGCTACTTCACGTTCTGGGTTGTTCTCTCGATCATCTGGGGTATCATGGCCACG  1950
 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    629
GGCGCGATGGTGCTGCTCCCCGTGTGGGAGTCGCGGTCGTCAATCCTCGGGGTTATTACTCTGGGTAAGATCGTACCG  2028
 g  a  m  v  l  l  P  V  W  E  S  R  S  S  I  L  G  V  I  T  L  G  K  I  V  P    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
TTGCGCATCAACGCAAACAGGTGGCAGACTTCGCGTGCGAAGTCCGCGTCCCATGCACGTTTTATTGATCGCCTCTGA  2184
TCGCTGCTTGTCCTAACTGCCCGGTGGGAGTGTGGGCCGGACACTCCCTGCCGCCTGCCCTTTTCGCCTAGCATACGG  2262
GAGGAGTGCTGTCGGTACTGTCACCGCTTCGTGCGGTGCGACGTTTCTTGTCACGTTGTCCTCATGTTGTCTCTCTTG  2340
                     <PyDUR3.3-GR
GCGGGGTAAGCTGGTTCACTATAGCAGGTTGTTGTCTAGTGGGTTATGTCAACCGTCACAAAAAAAAAAAAAAAAA    2416
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PyDUR3.3      MATpavvnpgiaawgcaat-vvrnfflDPQYECRESFFDNKP----VLDKWvgyvivlafgvafgiatvgivliEQRVLGRKMDSEFFNTAGRSVKTGLTASVIVSQ  102
PyDUR3.2        MADF...SVE.F..TTSP.T.........A.QA...GLADG-SAQ.Sra..........is.......l....R.I.N.P.....................l...  104
PyDUR3.1   MATSPA.IPGFNLPSHE..DSEAPW..ELDL...K.N....GDVSNPTHP.Ai.t..fvivgl..s...f..ll.AL..YLFSTV.T..Y............s.......  110
EsDUR3                               MTSVPED.IDQD...GGE.P----.SEA....v..i..if.sff.tfl.wlD.KFN.VSIT..Q.......i............   80
CrDUR3A                                            MRPRSAGDTA.PEGl.wf.ligl.av.alvptfllwaDRKFG.TQA...N.A.....i.a....cd..ak   70
AtDUR3                   MATCPPFDFSTKYYDG.GGCQRQS...GGTTV----..QG...avi.g..af.avf.sfl.wl.K.YV.ARHT..W......N.....i.......   92
ScDUR3                   MGEFKP-----------------------------P.PQGA..a...gl.av.agmm.lttyllK.YQKEIITA.E.T..........v.aav..s   67
                                                             *    *: ::  :*  *.          :        :* * ****:.*:** *: .*:.

PyDUR3.3   WTWAATLLQSSNVAFKYGVSgpfwyasgatlqiivfsllavqvKRRAPTAHTFLEIIQARWGTvahavflffalatnvivtsmlilGGSAVVSALtgistnlasfliplg  212
PyDUR3.2   ..................................i.a.............................t......f...i..............N.....n...........  214
PyDUR3.1   ...s..........YL..........a..sc.v.l.gm...l..l....S............R.P.t...v...t.s.......q...A...N....vnld...a...v.  220
EsDUR3     ...............N..............v.vll.gi..iE...K...C............DT..K.......l..i......l...a.T.N....ldl........w.  190
CrDUR3A    ..........A...WSF.........a...v.vll.ai...EI..K...I.......RC....T..Vi.v.ygfl..i...a......a..ME....v.vyas.....a.  180
AtDUR3     ......i.......WQ..............i.vll.gvm.iEI..K..N...VC..VK.....AT.I...v.c.....v..a..l.......N....vnlya........  202
ScDUR3     .i.cs...t..TKEYAD.if.gya..a..cf...a.ai..iKT.QM..N...Y..LVRT.Y.KIG.Gcy..y.i...il.....lts....FSD...mn.iasc..l.v.  177
           * *.:*:* *:.  :  *: * : **:**  *:: *.::*:  *  **. **  *::  *:*   *  :::: : *:: **:**  :*:*..  ***::   :: *:*:*

PyDUR3.3   vilytlagGLKATFvasyfntavilialcifvfqvyvTD-ATLGSPSAVYDRLQESVS-------------------FEPVVDNRGGSYLTMFSKNgllfglsnicgnfg  302
PyDUR3.2   .....i.................................-S......T......ATSV-------------------IQ..L..K.........................  304
PyDUR3.1   ..........R.............mlf.vt.s.v.ftQH-GDI...DKMWLL..RASK-------------------IV..EN.LD.....FW.RD..f...i.lv...s  310
EsDUR3     ..a..m........l...th.t.lfv..l....vi.TG.DQYV...G.MF.K..AL.KLDDCTYGPASDPMKYELYACG..DG.QE.....L.RE..fi..ii..v....  300
CrDUR3A    .vi..av....G.v..ewl.vc..y...l..m....A.N-KD...IKV.YE..TAVAEK-------------------H..A..M....M.....s.ii..ii..i....  270
AtDUR3     .vv...........l...vhsvivhv..vv...l..TSS-KE.....V.Y...KDM.AKSRS----CTEPLSHHGQACG..DG.FR......l.sg.av...i..v....  307
ScDUR3     .vv...f..I....ltd.mhtc..i.ivlv.a.k..A.S-DV....GK.Y.LVR.AAKR-------------------H..DG.YQ.E.M..T..Sagillii.li....  267
           *: **  **:: * ::: ::  ::: ::: * * ::       **   ::  :                          **  *  * *:*: *  :  : : *: ***:

PyDUR3.3   tvfvDQSYWQSAIAATPQAAWKGyilgglswfsipftlatslglagLALS---------LPITIDESNSGLVPPavathlmgkggsvlilimlfmavTSTGAAEQIAVSS  403
PyDUR3.2   ................................a..............i..---------....L..............................................  405
PyDUR3.1   ...i.............S.S...fl....c..a...........saV...---------..V.TA.AAR......a.g.fl.sa..avv.v.t.l....S..S.......  411
EsDUR3     ...............K..SSH...l...mc..t...a........a..MQ---------..L.G..AG.......T.LFVL...AT...............s..l.....  401
CrDUR3A    ..........G....K.S.TY...l...mc..a....f..tm..gaR..D---------..L.KA.A.K......l.va...q..af.vtfq......A.AC...M..AT  371
AtDUR3     .....NG..V.....R.SSTH...l....v..av..s.......ga...D---------...SK..ADR......T.IA....S..L.L.t........a.ss.l.....  408
ScDUR3     ...l.ng..NK..S.S.A.SL.A.AI...a..av.slisltm...c..vetSPNFPTYPD.L.SFQA.....l..a.iai....a.as.l.i.......AMS..L.....  377
           ***:*. **  **:*.* :: *::. **: **::*   ::: **.  *::          *::  ::  *** ** *  ::*::*:       *:***:: . *: **::

PyDUR3.3   LVAYDIYVPIRRHMGHNPTGKeiilvsriaivafgllmgvlgiaLNAIGVSLGwvylgmgviigsavapvamsiswAKCSGAgaisgavgglalaiaswlgyastFDG-G  512
PyDUR3.2   .....V.A.LM.R..KPV...svv......iv.....t.................................fa...S.......va..i...v...........s...-.  514
PyDUR3.1   I......R---T.INPRCS.PQV.f...vv.lt.....g.......H.....Dyl.rl...l.....f...f..t.GRA..V..vg..l...v.glst.....a.L..-.  517
EsDUR3     .iS..V.R---AYIKKDA..D..LK....tvfv..a....ls.v..EM.LN......f...v.....v..s.vlt.K.ATAK...a..il.qic..ig..vs.qaLE.-E  507
CrDUR3A    I......K.---YINK.AN..QM.ilq.vmv.vyaiis..isvi.lKLQ........f..iv.....f.i.a.lT......ata..vs..vttp..mt..it.AKLNDGV  478
AtDUR3     .fT....R---TYINPRA..RQ.Lki..c.vlg..cf..i.avv..KA......m..a...l.....i.i.fmll.S.Anaf...l..ts.cvfg.it..tt.K.QY.-R  514
ScDUR3     VFT....R---EYIDPRAS..Kl.yt.hv.cif...a.sgfsvg.YYG.i.m.yi.em..i..s...l..vlTLCSKDmnlva.vvspil.tg...m...vCTKSLYK-E  483
           : :**:*.     :     *  ::       : :    : : : *    :: :: *  **: * *** *:   :     .   *: . .      :  **: :

PyDUR3.3   VNIANTGLDQvmvvgnlvailssavic-avvsfiKPDNCDWSATKAIALVDEDVN-------------------------------------------------------  566
PyDUR3.2   .S.....R.E......v.......a..-v...l........L...S.P.L.A.A.-------------------------------------------------------  568
PyDUR3.1   I.LET..NTD..la...ms....gl..-tli..vR..D....S.QS.P.IED.P.-------------------------------------------------------  571
EsDUR3     IT.DS..QNYp.lt...i..cf.giv.-ia..laD..DY..ES.RN..M.ETYD.-------------------------------------------------------  561
CrDUR3A    I.LDT..Q.YP.la.....lff.mil.-iil.y.f.QDF..AELRN.PVIEC.P.S------------------------------------------------------  533
AtDUR3     .DLDS..KNGP.LA.......T-----------VR.Q.Y...T.RE.KV.EAYASGD-----------------------------------------------------  560
ScDUR3     LTVDT.FM.YP.lt.....l..p.ifipiltyvf..Q.F..EKM.D.TR...TAELVQADPDIQLYDAEANDKEQEEETNSLVSDSEKNDVRVNNEKLIEPNLGVVISNA  593
           : : .*     *::**:::::               *:: **  :  * ::   .

PyDUR3.3   ---------AELSPEDEAEIDRAMKMISAwgiglalvlvvawpllalpag-vfSKGyftfwvvlsiiwgimatgamvllPVWESRSSILGVITLGKIVPTPEPKSLEDSL  666
PyDUR3.2   ---------...T..A..Q..K.....T...v..................-...........i......l...i...............AILSC.A.S.PLPV.TM.EPV  668
PyDUR3.1   ---------.F.PF.RAEALE..F.Rvagf.vlitali..v........R-...RS..A.yai...a..vi.asi.iv.......Q...V.L...MYARRL.AAEAAKDS  671
EsDUR3     -------AWHTEADYNDVDLSK.KNW.Ikv.lsftv.i.il..v.s....-...E...d...a...l..lv..mfv.f..LY..KDA.VS.FKGMCGQAPAGKTVQQS.Q  663
CrDUR3A    ------DPNNFEGEDSPEALNHVi.ftw.t.gt.t...lil..i.....K-...E......iiiam....v.scvciv..I..ASDV.VNFF.WK.APVAGGVATTAEES  636
AtDUR3     -----EDVDVPAEELR.EKLR..KAW.vk..lvftili..i..v.s...R-...R...w..aiva.a..tigsiviig..LV..WDT.KS.CMGMFTNDRVMK.LDDLNH  664
ScDUR3     IFQEDDTQLQNELD.EQR.LA.GL.iayflcvff..afl.v..mpMYGSKYI...KF..g...vm...lffsaf.vciy.l..G.HG.YTTLRGLYWDLSGQTYK.REWQ  703
                              : :           ::  ::: **:    :  :**. :*  :  : : *  ...      *: *.   *

PyDUR3.3   ELAESADGESTNE                                                          679 (-----, 15 TDs)
PyDUR3.2   GESDGISS.VDE                                                           680 (80.7%, 15 TDs)
PyDUR3.1   FHESGGGS.AGD.EGGYEVGYADGFAAARAAVAAGSGMPLDGELPDLMGPDGGGHRLVEPPEPSHRTSF  740 (56.7%, 15 TDs)
EsDUR3     QAPSA                                                                  668 (54.0%, 15 TDs)
CrDUR3A    AHKGDHMELPPAPANRA                                                      653 (47.8%, 15 TDs)
AtDUR3     R.RALTMAVPEA.KIYLLELEKTKKNDEEG                                         694 (49.3%, 14 TDs)
ScDUR3     NSNPQDLHVV.SQISARAHRQSSHFGQVDEII                                       735 (36.8%, 15 TDs)
```
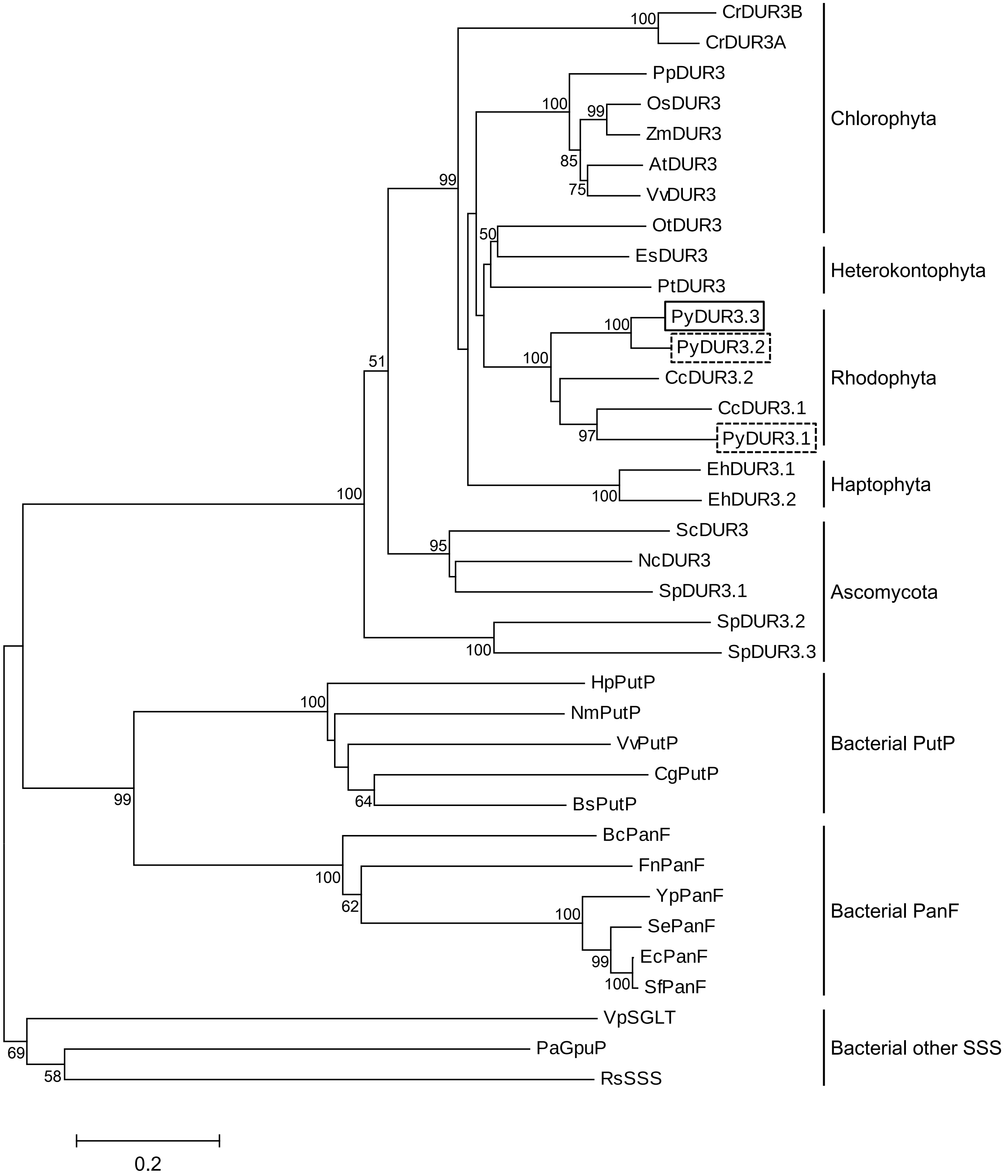
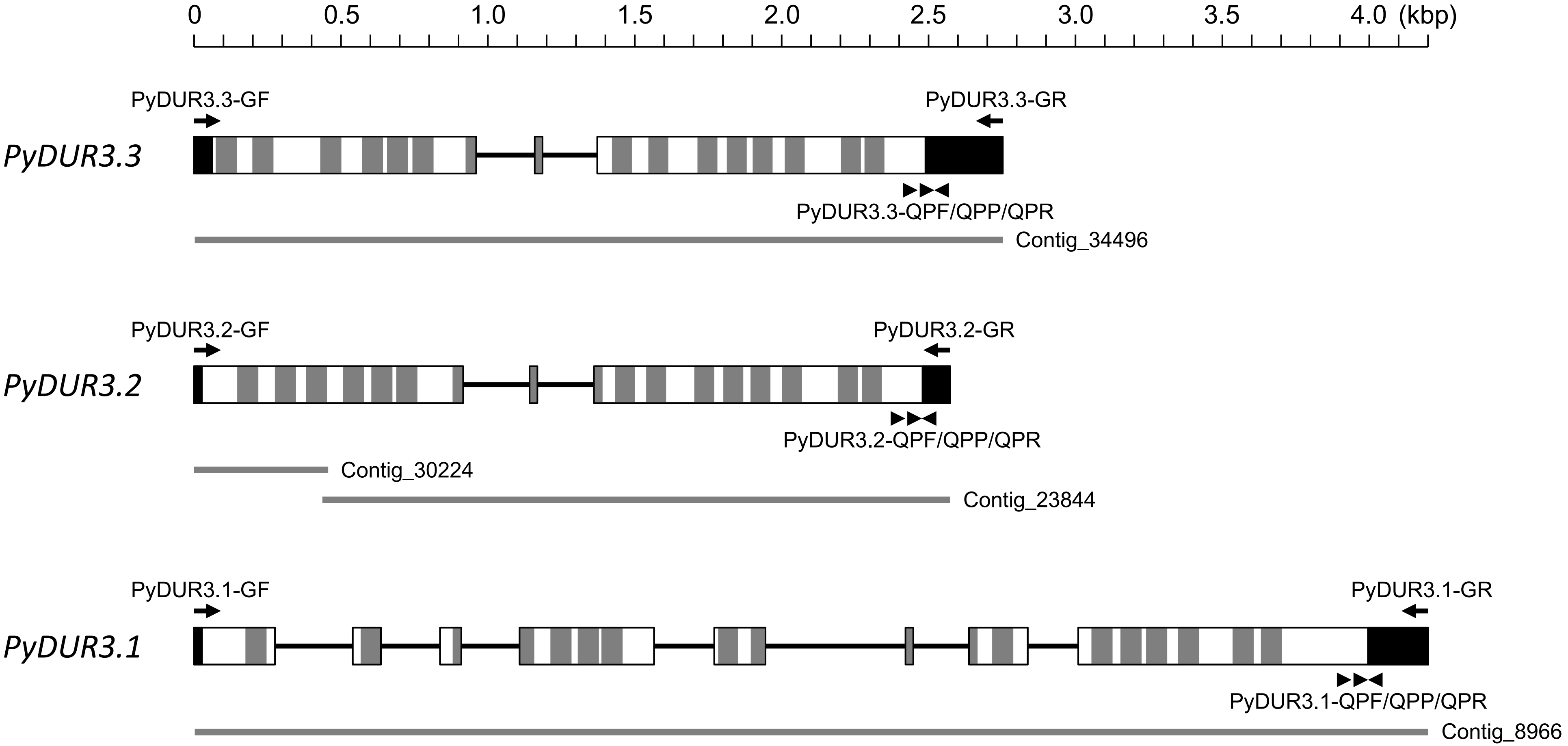
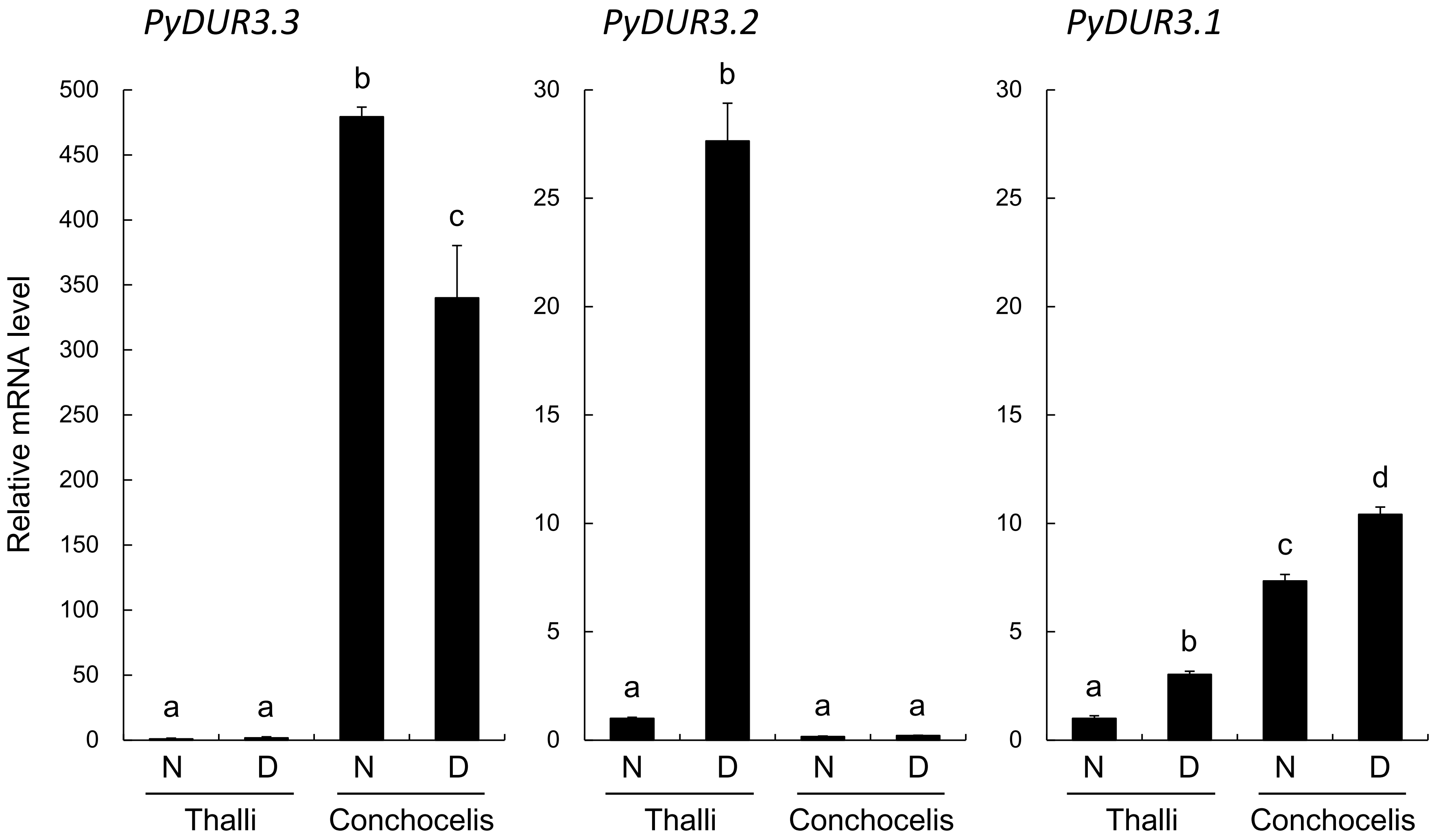

Kakinuma et al., Fig. 2

Kakinuma et al., Fig. 3





Kakinuma et al., Fig. 4



Kakinuma et al., Fig. 5