Molecular characterisation and function analysis of the rice OsDUF872 family

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ABSTRACT
With the advance of sequencing technology, the number of sequenced plant genomes has been rapidly increasing. However, many plant proteins in public databases are recognised as proteins with domains of unknown function (DUF). DUF872 is such a protein family that consists of plant proteins with unknown function. In this study, we analysed three DUF872 members (OsDUF872.1, OsDUF872.2 and OsDUF872.3) in rice Nipponbare with three distinct motifs. Real-time polymerase chain reaction showed that the expression patterns of the three corresponding OsDUF872 protein-encoding genes varied in 15 different rice tissues. The expression of OsDUF872.2 was significantly (P < 0.01) upregulated under salt, cold and heat stress conditions. Overexpression of OsDUF872.2 in Escherichia coli significantly improved the resistance to heat. These results improve our understanding of these poorly-studied proteins and provide information for future studies on proteins of unknown function.

Introduction
Plants are subject to various abiotic stress conditions, including heat, cold, drought and salt, during their life cycles, which may adversely affect the productivity of plants in agriculture. In order to counteract these negative effects, plants have developed a number of physiological and biochemical strategies that can sense stress signals and help plants to adapt to adverse environments [1–3]. Heat stress, one of the restricting factors, usually affects plant growth, seed germination, photosynthesis, respiration and membrane stability [4]. The temperature increases associated with global warming rapidly increase. However, many plant proteins in public databases are recognised as proteins with domains of unknown function (DUFs) [20]. The names of DUFs are given when protein families have no functional annotation in the Pfam database (http://pfam.xfam.org/family) [21]. Recent studies have demonstrated that some DUF-containing proteins could enhance the tolerance to stresses in rice [22,23]. The DUF872 family consists of plant proteins that contain DUFs and have an average length of around 108 residues. To our
knowledge, none of the members in this protein family have been functionally characterized in rice.

In the present study, we analysed the protein sequences of the OsDUF872 family in rice, their spatio-temporal expression profiles and their expression patterns under various stresses and abscisic acid (ABA). Furthermore, we overexpressed OsDUF872.2 in Escherichia coli and found that OsDUF872.2 may improve the heat resistance. This result may provide new insights into the function of the OsDUF872 family in rice. All the results obtained in this study improve the understanding of the poorly-studied OsDUF872 proteins and provide information for future studies on other proteins of unknown function.

Materials and methods

Database searches
The sequences of putative OsDUF872 family proteins in rice were obtained by searching the rice genome annotation project (RGAP version 7) database (http://rice.plantbiology.msu.edu/) with domain number PF05915. All the corresponding sequences of the putative OsDUF872 family members were then downloaded from RGAP and confirmed with the SMART database (http://smart.embl-heidelberg.de/smart/batch.pl) [24,25]. If there were several gene models at one locus, only the complete gene model was selected for further sequence analysis.

Sequence analysis
Information about the chromosomal localization, amino acid (aa) sequence length and full length cDNA accesses was obtained for each OsDUF872 gene from RGAP (http://rice.plantbiology.msu.edu/). The protein localisations of OsDUF872 members were predicted using the online protein location prediction software TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) [26]. MEME version 4.9.1 (http://meme.nbcr.net/meme/) was used to predict the motifs of OsDUF872 family members [27]. Alignment of the protein sequences of OsDUF872 family members was performed using Clustal Omega [28]. A phylogenetic tree was constructed using MEGA4 and bootstrap testing was performed with 1,000 resamplings [29].

Plant growth and various stress and ABA treatment
Rice (Oryza sativa L. subsp. japonica cv. Nipponbare) seeds were germinated for 3 days, and then seedlings were grown hydroponically in a 30-L vessel containing nutrient solution [30]. At the emergence of the fourth leaf, the seedlings were subjected to various stresses and ABA treatment. For high salinity treatment, NaCl solution was added to achieve a final concentration of about 200 mmol/L. Drought stress was modelled by putting intact plants in the air without water supply. For cold stress, the vigorous seedlings were transferred to a growth chamber at 4 °C (12-h-light/12-h-dark cycle). For heat stress, the seedlings were subjected to 42 °C heat shock treatments. For ABA treatment, 100 µmol/L ABA was sprayed onto the leaves of seedlings.

RNA extraction and real-time polymerase chain reaction (PCR)
Samples were collected and ground to fine powder in liquid nitrogen. Total RNA was extracted with Trizol reagent (GIBCO, Burlington, ON, USA) according to the manufacturer’s instructions. Next, the total RNA was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) for 15 min to degrade any potential DNA contamination, and then used for first-strand cDNA synthesis. Real-time PCR was performed as described before [31]. The rice Actin1 gene (LOC...Os03g50885) was used as an internal control with primers 5'-TGGCATCTCTCACATTCC-3' and 5'-TGCAATGGGTCGCGAGA-3’. The primers used in the expression analysis of the OsDUF872 gene family members are listed in Table S1 in the Supplementary Appendix. The 2^(-ΔΔCt) method was used for analysis of the relative expression data as described previously [32]. All the quantitative PCR products have been confirmed by sequencing.

Assay for heat stress tolerance of E. coli transformants
In order to construct the expression vector pET32a-OsDUF872,2, specific primers were designed as follows: sense, 5'-GGATCCATGGCGTCTAGACGCAATGT-3' (BamHI site underlined), and antisense, 5'-AAGCTT TTAGTGTAATAAAATAAGA-3' (HindIII site underlined). The amplified products were cloned into a pET32a vector at the BamHI-HindIII site to express the fusion protein, which has a Trx•Tag™ thioredoxin at the N-terminus [33]. The transformed E. coli Rosetta cells (Sanborn, MN, USA) were grown in Luria–Bertani (LB) liquid medium containing 100 µg/mL of ampicillin at 37 °C overnight, then they were inoculated into fresh LB medium (1:100 dilution) supplemented with ampicillin (100 µg/mL) to be incubated until the exponential growth phase (optical density at 600 nm (OD_{600}) of 0.5–0.6). Isopropylthio-β-D-galactoside (IPTG) was added into the cultures to induce the expression of the transformed gene. For the heat tolerance assay, 2-mL samples were placed into a 50 °C
water bath. At 0, 0.5, 1, 1.5, 2 and 2.5 h after heat shock, respectively, 100 µL of different dilutions (1:100) were spotted onto LB agar plates with 1 mmol/L IPTG.

**Data analysis**

The experiments were repeated three times. Data are mean values from three independent experiments, with standard error of the means. Statistical analysis was performed using the Student’s t-test by EXCEL version 2003.

**Results and discussion**

**Identification of OsDUF872 family members in rice**

Three genes encoding OsDUF872 family proteins were obtained (Table 1) by searching the rice genome annotation project using the domain number PF05915. The presence of these OsDUF872 genes in the rice genome was then confirmed with the SMART database (Figure S1 in the Supplementary Appendix). These genes were named as OsDUF872.1 to OsDUF872.3 according to their positions on pseudomolecules. OsDUF872.1 is located on chromosome 2, OsDUF872.2 is on chromosome 11 and OsDUF872.3 is on chromosome 12. The rice genome includes 10 duplicated blocks accounting for 45% of its total size [34]. Therefore, it could be speculated that genome duplication might be the mechanism that underlies the distribution of OsDUF872 family members in different chromosomes, similar to that previously suggested for OsDUF866 family members and OsDUF946 family members [31,35].

**Sequence analysis of OsDUF872 family proteins**

Information about the amino acid length, full-length cDNA accessions, molecular weights (MW) and isoelectric points (pI) is presented in Table 1. The length of the OsDUF872 proteins was predicted to vary from 104 aa (OsDUF872.1) to 226 aa (OsDUF872.3). The putative OsDUF872.3 was predicted to be located in the chloroplast, whereas OsDUF872.1 and OsDUF872.2 were both predicted to be located in locations other than chloroplasts and mitochondria.

The multiple sequence alignment analysis of the OsDUF872 members was performed using Clustal Omega (Figure S2 in the Supplementary Appendix). The results showed that the percent identity was 17.65% for OsDUF872.1 and OsDUF872.2, 31.00% for OsDUF872.1 and OsDUF872.3, and 67.20% for OsDUF872.2 and sDUF872.3. The MEME motif search tool (http://meme.sdsc.edu/meme/) was applied on the three OsDUF872 sequences and three distinct motifs were identified (Figure 1). One motif 1, one motif 2 and one motif 3 were found in OsDUF872.3. The predicted OsDUF872.2 sequence includes one motif 1 and one motif 2 but no motif 3, whereas the OsDUF872.1 sequence includes only one motif 3 but no motif 1 and motif 2.

In order to examine the evolutionary relationships among the OsDUF872 family members in rice and Arabidopsis, a phylogenetic analysis was performed using MEGA4. Here three Arabidopsis DUF872 members, AT2G19350.1, AT3G29170.1 and AT4G29850.1, which were all predicted to be located in the plasma membrane, were included as reference sequences. The results indicated that the putative OsDUF872 proteins could be classified into three major groups (I, II, III) (Figure 2). Group I contained AT2G19350.1, AT4G29850.1 and OsDUF872.1, group II contained only one member AT3G29170.1 and group III contained OsDUF872.2 and OsDUF872.3.

Expression patterns of OsDUF872 family members in various tissues

The expression patterns of OsDUF872 family members in various tissues of rice at different stages remains poorly understood so far. Therefore, we examined the expression of the OsDUF872 family members in 15 different tissues by real time PCR. The results indicated that the spatio-temporal expression patterns of the three OsDUF872 members were completely different (Figure 3). For OsDUF872.1 and OsDUF872.3, the highest expression was in the stem at the ripening stage, whereas the lowest expression was in the leaf sheath from plants with four tillers. For OsDUF872.2, the highest expression was in the stem from plants with four tillers, whereas the lowest expression was also in the leaf sheath from plants with four tillers. These results suggested that the three OsDUF872 family members could play different roles in all tissues at different stages in rice.

| Name     | MSU locus   | FL-cDNA accession no. | AA length | Introns | MW     | pI | Location        |
|----------|-------------|------------------------|-----------|---------|--------|----|----------------|
| OsDUF872.1 | LOC...Os02g07980 | AK062746               | 104       | 1       | 10 802.5 | 6.51 | Other location |
| OsDUF872.2 | LOC...Os11g03760 | AK059074               | 139       | 2       | 15 896.2 | 6.228| Other location |
| OsDUF872.3 | LOC...Os12g03500 | AK243479               | 226       | 2       | 25 638  | 11.24| Chloroplast   |

Table 1. OsDUF872 genes and the properties of the putative proteins they encode.
Expression analysis of OsDUF872 genes under various stress conditions

The expression of the three OsDUF872 family members under cold, heat, salt, drought stress conditions and ABA treatment in Nipponbare rice seedlings was examined at the emergence of the fourth leaf (Figure 4). The expression of OsDUF872.1 was significantly (P < 0.01) upregulated under salt conditions. The expression of OsDUF872.2 was significantly (P < 0.01) upregulated under salt, cold and heat conditions, yet downregulated under drought conditions. OsDUF872.3 showed significantly (P < 0.01) downregulated expression under drought, salt and heat stress conditions.

In general, members of the same gene family tend to have similar behaviour under the same stress conditions [36–38]. However, in this study, we found that the three OsDUF872 family members in rice displayed very different expression patterns under various stress conditions and ABA treatment, suggesting that OsDUF872 genes play different roles in response to various stresses.

OsDUF872.2 improved heat resistance in transgenic E. coli

Since the expression level of OsDUF872.2 was significantly elevated under heat stress conditions, as described above, we hypothesized that it may be involved in the response to heat stress. To test this hypothesis, we overexpressed this gene in E. coli and examined the heat stress resistance.

Under normal conditions, there was no evident difference in the number of colonies between transgenic lines (E. coli transformed with pET32a-OsDUF872.2), and the control (E. coli transformed with pET-32a), whereas under heat conditions, the number of transgenic colonies (Rosetta/OsDUF872.2) was much higher than that in the control (Rosetta/pET-32a) at 0.5, 1, 1.5, 2 and 2.5 h after heat treatment, respectively (Figure 5). These results indicated that the overexpression of OsDUF872.2 in E. coli may significantly improve the resistance to heat.

At present, the specific mechanism by which heat tolerance is enhanced in E. coli recombinants transformed with OsDUF872.2 remains unclear. It has been reported that the overexpression of some heat shock protein genes can enhance the heat tolerance in E. coli [39–42].
Under heat conditions, it is possible that OsDUF872.2 interacts with some heat shock protein, and the protein–protein interaction can lead to improve the heat stress resistance in transgenic *E. coli*. Another possibility is that the heat stress signal was strengthened in *E. coli* recombinants transformed with OsDUF872.2, and the enhanced heat stress signal promotes the increased expression of a number of heat stress-related genes, leading to improved heat tolerance in transgenic *E. coli*.

The functions of some DUF proteins have been elucidated recently. Many DUF-containing proteins are involved in plant stress response. TaSRG, a wheat DUF662 domain-containing transcription factor, significantly affects salt tolerance in transgenic *Arabidopsis* [43]. OsDSR2, encoding a protein with a DUF966 domain, negatively regulates the rice response to salt and simulated drought stresses as well as ABA signaling [22]. OsSIDP366, a DUF1644 gene, positively regulates the salt and drought resistance in rice [44]. In our previous study, OsDUF866.1 improved heat stress resistance [31] and OsDUF946.4

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**Figure 3.** Real time PCR analysis of OsDUF872 genes in different tissues of *Nipponbare* rice. Fifteen representative tissues are as follows: Lb1, leaf blade at four-leaf stage; Lb2, leaf blade from plants with four tillers; Lb3, leaf blade at ripening stage; Ls1, leaf sheath at four-leaf stage; Ls2, leaf sheath from plants with four tillers; Rt1, root at four-leaf stage; Rt2, root from plants with four tillers; St1, stem from plants with four tillers; St2, stem at ripening stage; An, 1.2–1.5 mm anther; Pi, pistil from 10–14 cm inflorescence; Em1, embryo at 7 days after flowering; Em2, embryo at 28 days after flowering; En1, endosperm at 7 days after flowering; En2, endosperm at 28 days after flowering.

Note: The rice *Actin1* transcript levels were used as internal controls. Error bars indicate standard error of the means based on three biological replicates.

**Figure 4.** Relative expression levels of OsDUF872 genes in *Nipponbare* rice seedlings at the emergence of the fourth leaf under various stress conditions and ABA treatment detected by real time PCR. D, drought; S, salt; C, cold; H, heat; A, ABA.

Note: For salt stress and drought stress, seedlings were sampled at 0, 4, 8 and 16 h. For ABA, heat and cold stress, seedlings were sampled at 0, 1, 3, 8 h. The rice *Actin1* transcript levels were used as internal controls. Error bars indicate standard error of the means based on three biological replicates. **, *P* < 0.01 (Student’s *t* test).
improved the tolerance to salt and drought in transgenic *E. coli* [35]. In this study, OsDUF872.2 improved the heat resistance in transgenic *E. coli*. In order to improve plant abiotic stress resistance, we can consider the DUF family members as a genetic resource of high potential.

**Conclusions**

This study provided not only important sequence information regarding the OsDUF872 family members, but also showed the expression patterns of OsDUF872 family members in different tissues, and under various abnormal conditions including abiotic stress and ABA. Furthermore, this study suggested that OsDUF872.2 could improve the resistance to heat stresses in transgenic *E. coli*. All these data can provide important reference for further studies of the functions of OsDUF872 family and contribute to the genetic improvement of rice.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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