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Research paper

Genome-wide analysis and expression profiles of NTMC2 family genes in *Oryza sativa*

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ABSTRACT

N-terminal-TM-C2 domain proteins (NTMC2), which share domain architecture and sequence similarity to synaptotagmins (Syts) in mammals and FAM62 (extended Syts) in metazoans, form a small gene family in plants. Previous studies showed that the *Arabidopsis thaliana* NTMC2 type 1.1 protein (NTMC2T1.1, named AtSyt1) possesses calcium- and membrane-binding activities that allow it to function in a plasma membrane repair pathway induced by stress. However, we lack understanding of the diverse biological roles of plant *NTMC2* family genes. In this study, a total of 13 *OsNTMC2* genes was identified through a comprehensive bioinformatics analysis of the rice (*Oryza sativa* L.) genome and classified into six *OsNTMC2* groups (*OsNTMC2T1* to *OsNTMC2T6*) based on phylogeny and motif constitution. OsNTMC2T1 to OsNTMC2T3 have two calcium-binding domains (C2A and C2B), but OsNTMC2T4 to OsNTMC2T6 have single C2 domain. The expression profiles of *OsNTMC2* genes were analysed at different stages of vegetative and reproductive development. This analysis revealed that at least one *OsNTMC2* gene was abundantly expressed at each stage of development. These results should facilitate research on this gene family and provide new insights elucidating their functions in higher plants.

1. Introduction

Synaptotagmin proteins (Syts) form a group of membrane-trafficking proteins, each with an N-terminal transmembrane (TM) sequence followed by a variable-length linker and two tandem distinctly conserved calcium-binding domains (C2A and C2B) in the C terminus (NTMC2). Syts are widespread in eukaryotes. Domain architecture and sequence similarity to the Syt C2 domain are also shared by the evolutionarily conserved FAM62 gene family, found in metazoans. The NTMC2 gene family in plants comprises six gene types that have a unique, conserved gene structure. Different plant species have different numbers of NTMC2 family genes. All Syts and extended Syts (Esyts) genes in plants are grouped to six types of NTMC2 gene: NTMC2 type 1 (NTMC2T1) to NTMC2 type 6 (NTMC2T6). Each of these six types has its own conserved gene structure, but little is known about the function of the FAM62 gene and the Esyts gene family.

Syt I was first identified as an abundant synaptic-vesicle integral membrane protein with a calcium-dependent phospholipid-binding protein (Perin et al., 1990; Geppert et al., 1994). The other members of the Syt gene family have subsequently been discovered by DNA sequence similarity. Syt II was reported to be highly homologous to Syt I. RNA blots demonstrated complementary patterns of expression for Syt I and II, with Syt I expressed preferentially in rostral, phylogenetically younger brain regions, and Syt II expressed predominantly in caudal, phylogenetically older brain regions (Geppert and Südhof, 1991). RNA blotting studies also revealed that Syt III mRNA is expressed in the brain, various endocrine tissues, and hormone-secreting clonal cells (Mizuta et al., 1994). Syt V is expressed at high levels in rat brains, but not in the spinal cord nor in a number of peripheral non-neuronal tissues (Craxton and Goedert, 1995). Synaptotagmin V mRNA is expressed in rat kidney, adipose tissue, lung, and heart, as well as at higher levels in the brain and PC12 cells (Hudson and Birnbaum, 1995). Syt IV, VI, VII, and VIII are also expressed widely in non-neural tissues. Although the expression levels of Syt VI and Syt VII are highest in the brain, Syt VII is also abundant in the heart and lung (Li et al., 1995). *Syt X* was reported to be induced by kainic acid, a cyclic analogue of glutamate, with elevation at 3 h, strong induction at 6 h, and a return to the basal level by 12 h after kainic acid administration to the hippocampus. In

Abbreviations: qRT-PCR, real-time quantitative RT-PCR; Ct, cycle threshold; UBQ, ubiquitin; Syt, synaptotagmin; Esyt, extended synaptotagmin protein; NTMC2, N-terminal transmembrane and C2 domain; TM, transmembrane; SMP, synaptotagmin-like mitochondrial membrane protein; TRAP-Seq, translating ribosome affinity purification followed by mRNA sequencing

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the piriform cortex, smaller degrees of *syt X* induction were observed at 3 and 6 h after kainic acid administration. The piriform cortex also appeared to have a higher level of constitutive *syt X* expression compared with the hippocampus and parietal cortex (Babity et al., 1997). *Syt XI* is expressed highly in the brain and at lower levels in the spleen, lung, liver, skeletal muscle, kidney, and testis (von Poser et al., 1997). Similar to *syt III*, *IV*, *VI*, *VII*, *IX*, and *XI*, *syt XIII* is expressed most highly in the brain and at lower levels in non-neuronal tissues (Südhof, 2001). Unlike expression of other Syt isoforms, the expression of *Syt XIV* in mice is restricted to the heart and testis; it is absent from the brain, where most other Syts are expressed abundantly (Fukuda, 2003b). In addition, mouse and human Syt XV, unlike other Syt isoforms, each have an alternative splicing isoform that lacks the C-terminal portion of the C2B domain (named Syt XV-b). Syt XV-a/b mRNA was expressed mainly in non-neuronal tissues (e.g. lung and testis) (Fukuda, 2003a). Syts VIII, XII, XIII, and XIV are Ca^{2+}-independent Syts capable of forming a Ca^{2+}-independent oligomer. Such diversity among structures and expression patterns reflects the diverse roles and flexibility of Syts.

The literature contains little information about the possible functions of FAM62 and plant syt genes. FAM62 proteins (also called Esyts) were first reported in 2007, and the primary structures and biochemical properties of members of this evolutionarily conserved mammalian protein family have been analysed (Min et al., 2007). In 2009, the HUGO Gene Nomenclature Committee changed the provisional nomenclature for FAM62 genes to Esyt (Craxton, 2010). Plant synaptotagmin-like genes were reported in 2001. Four Syts genes were identified in *Arabidopsis thaliana* by using TBLASTn to probe DNA sequence databases with a consensus peptide sequence corresponding to the most highly conserved region of the rodent Syt gene family, which is within the C2B domain (Craxton, 2001). The first two independent studies dealing with the functional role of synaptotagmin 1 protein (AtSYT1; AtNTMC2T1.1) were reported on in 2008 (Schapire et al., 2008). Loss of function of AtNTMC2T1.1 reduces the viability of cells due to a decrease in the integrity of the plasma membrane. This reduced integrity is enhanced in the syt1-2 null mutant under osmotic stress, likely due to defective plasma membrane repair (Schapire et al., 2008). Calcium-dependent freeze tolerance was inhibited by the extracellular addition of an antibody against the cytosolic region of AtNTMC2T1.1. Protoplasts and leaf sections isolated from *AtNTMC2T1.1*–RNA interference plants lost calcium-dependent freeze tolerance compared with wild-type plants (Yamazaki et al., 2008). Lewis and Lazarowitz (2010) further showed that AtNTMC2T1.1 regulates endosome recycling and movement protein–mediated trafficking of plant virus genomes through plasmodesmata. Subsequently, Yamazaki et al. (2010) transiently expressed a series of truncated proteins in protoplasts and determined that tandem C2A-C2B domains are necessary for the localisation of AtNTMC2T1.1 to the plasma membrane. *Arabidopsis* NTMC2T1.2 (AtSyt2) is expressed mainly in *Arabidopsis* pollen and plays an important role in the regulation of pollen germination and pollen tube growth (Wang et al., 2015). Localisation studies showed that AtNTMC2T1.2 is present on the Golgi apparatus (Zhang et al., 2011) and can be delivered to the plasma membrane in *Arabidopsis* suspension cells via a conventional pathway (Wang et al., 2015). The rice NTMC2 gene was first reported in 2004 (Craxton, 2004), but its function in plants has not been examined. In this study, we examined the diverse structures and expression profiles of rice NTMC2 genes at different developmental stages to identify developmental stages in which this gene family is involved and in which these genes are required.

2. Materials and methods

2.1. Database search for NTMC2 genes in the rice genome

A BLAST search was performed in the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu) (Kawahara et al., 2013) using known *AtSyts* gene cDNA sequences (http://www.arabidopsis.org/) as queries. The BLAST E-value threshold was 1e-5. We screened out 13 C2 domain–containing proteins with high degrees of homology with *AtSyts* in the rice genome (Additional file 1).

2.2. Phylogenetic analysis of the NTMC2 gene family

To investigate the evolutionary relationships of *NTMC2* genes, a phylogenetic tree was generated from the deduced amino acid sequences of 11 *AtNTMC2* genes from *Arabidopsis thaliana* and 13 *OsNTMC2* genes from *Oryza sativa*. The unrooted phylogenetic trees were constructed using the MEGA 7 program (Kumar et al., 2016) with the maximum-likelihood (Jones et al., 1992) and neighbour-joining (Saitou and Nei, 1987) methods.

Exon–intron structure analysis was performed using GSDS (http://gsds.cbi.pku.edu.cn) (Guo et al., 2007). Prediction of transmembrane helices in OsNTMC2 proteins was performed using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.3. Digital expression analysis

RNA sequence data were used to analyse the expression profiles of *OsNTMC2s* in rice tissues during different developmental stages. FPKM (fragments per kilobase of exon per million fragments mapped) values of leaves 20 days after germination, post-emergence inflorescences, pre-emergence inflorescences, panicles, anthers, pistils, seeds 5 days after pollination, seeds 10 days after pollination, embryos 25 days after pollination, endosperm 25 days after pollination, shoots, seedlings, and callus of RNA-Seq data, and FPKM values of panicles, seedlings, and callus of TRAP-Seq (the translating ribosome affinity purification followed by mRNA sequencing) were downloaded from the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu). The results are shown as a heatmap with hierarchical clustering. R x 64 v. 3.2.1 was used to generate the heatmap. The FPKM values of rice NTMC2 genes in panicles, seedlings, and callus from RNA-Seq and TRAP-Seq were also compared.

Genome-wide microarray data were used to analyse the expression profiles of OsNTMC2s in organs during different developmental stages (GSE6893) and under abiotic stresses (GSE6901). The average log signal values of OsNTMC2 genes in various developmental stages and fold-change values of up- and down-regulated genes under drought stress, salt stress, and cold stress were downloaded from the website http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi. The results are shown in a histogram.

2.4. Plant materials and real-time quantitative RT-PCR analysis

The rice cultivar Zhonghua15 was used for quantitative expression analysis. Seven tissues from throughout the rice life cycle (Additional file 2) were collected as described previously (Ye et al., 2009). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses were performed for every OsNTMC2 family gene. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesised using the SuperScript first-strand synthesis kit (Invitrogen). Real-time PCR was performed in an optical 96-well plate, including 12.5 μl 2 ∗ SYBR Green Master mix reagent (ABI, USA), 1 μl cDNA, and 0.2 mM of each gene-specific primer in a final volume of 25 μl, using the following thermal cycle: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min. Dissociation curve analysis was performed as follows: 95 °C for 15 s and 60 °C for 1 min. The UBQ5 gene was used as an endogenous control to

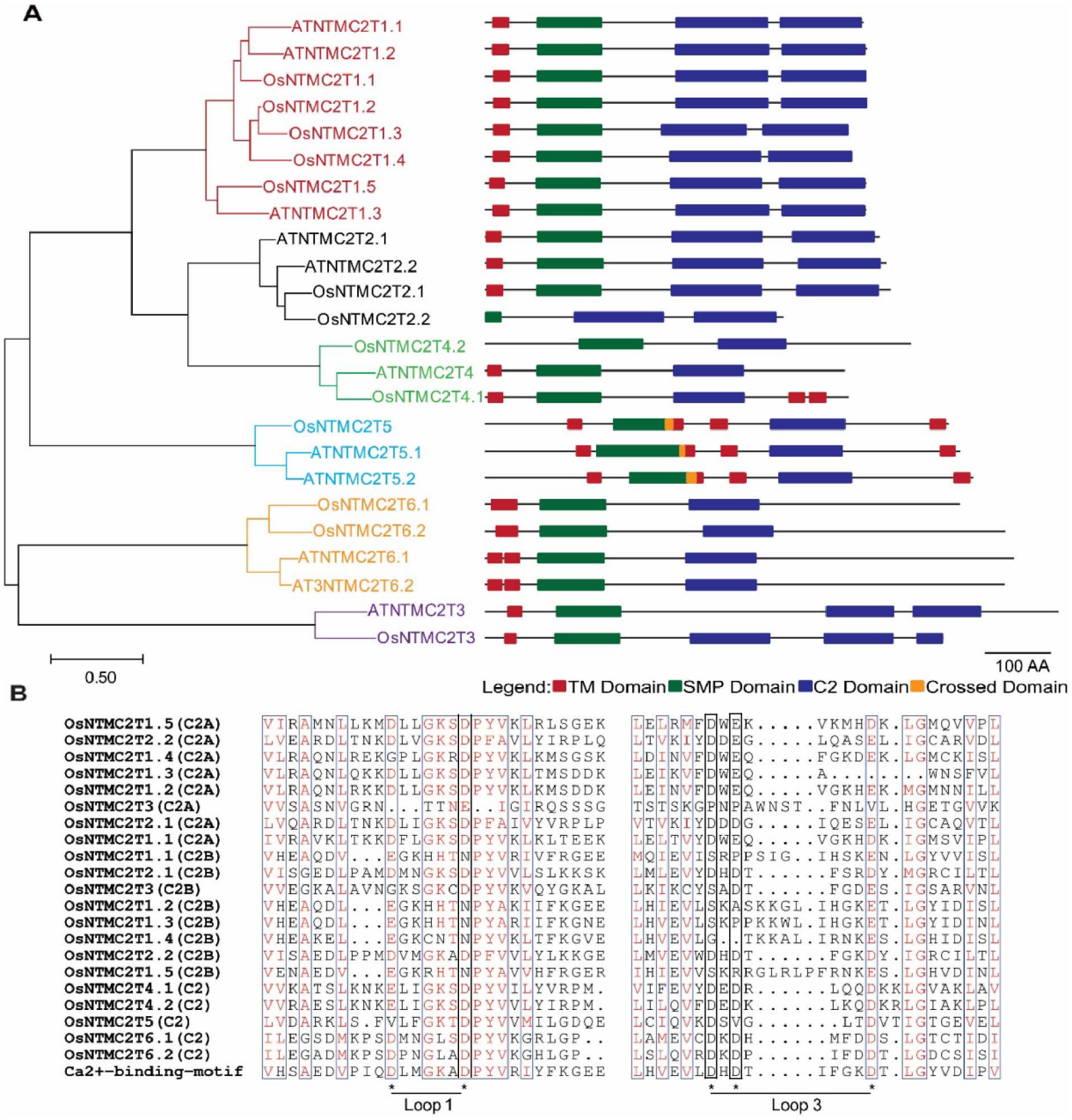


Fig. 1. Conservation of NTMC2s in *Oryza sativa* and *Arabidopsis*.

(A) Phylogenetic tree constructed using the amino acid sequences collected from *Oryza sativa* and *Arabidopsis*. Thirteen OsNTMC2s and 11 AtNTMC2s were analysed. The tree was generated using MEGA software (ver.7) with the maximum likelihood algorithm. The scale bar indicates the number of amino acid substitutions per site. Subfamilies are indicated by separate colours and named according to plant nomenclature with Arabic numerals. Subfamilies were defined by major branches in the diagram and consist of members that are more highly conserved across different species than to other members within a particular species. The domain structures of NTMC2s are shown. The C2 domain and synaptotagmin-like mitochondrial membrane protein (SMP; DUF 2404) domain were searched in NCBI using the Conserved Domain Search Service. TM domains were analysed using TMHMM Server v. 2.0. These domains are indicated by separate colours.

(B) Multiple alignment of the C2 domains of OsNTMC2s, in which OsNTMC2T1s, OsNTMC2T2s, and OsNTMC2T3s have C2A and C2B domains. The alignment was generated using the ClustalW2 algorithm and presented using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Asterisks indicate the conserved amino acid residues in the calcium-binding motif (loops 1 and 3) in the C2 domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

normalise the expression levels of the OsNTMC2 genes (Jain et al., 2006). All amplicons in these determinations followed the amplicon design criteria and were modified based on the primer Tm value. The data were analysed using ABI 7500 system SDS software, and the Ct value was exported and analysed according to the comparative Ct method (Schmittgen and Livak, 2008). The primers for every gene are listed in Additional file 3. Three biological replicates were performed for this experiment.

3. Results

3.1. Phylogenetic conservation of NTMC2s in Oryza sativa and Arabidopsis thaliana

Using the *AtNTMC2T1.1* full-length cDNA sequence as a query to blast the MSU Rice Genome Annotation Project Database and *Arabidopsis* Genome Database, we found 13 C2 domain–containing

proteins with high homology to *AtNTMC2T1.1* in the rice genome and 11 AtNTMC2s in the *Arabidopsis* genome (Additional file 1). An unrooted phylogenetic tree generated from the alignments of these 24 *NTMC2* protein sequences using the maximum-likelihood method (Fig. 1A) and the neighbour-joining method (Additional file 4) showed that these *NTMC2* proteins could be classified broadly into six major clades, presented in different colours (NTMC2T1–6). NTMC2T1 contained five OsNTMC2s and four AtNTMC2s. NTMC2T2 contained four members, two from each species. NTMC2T3 contained one OsNTMC2 member and one AtNTMC2 member. These three types of *NTMC2* gene belong to the previously named *Syt* family. The other three types of NTMC2, NTMC2T4–6, belong to the *Esyt* family. NTMC2T4 contained two *Oryza sativa* members and one *Arabidopsis thaliana Esyt* member. NTMC2T5 contained one *Oryza sativa Esyt* member and two *Arabidopsis thaliana Esyt* members. NTMC2T6 contained two *Oryza sativa Esyt* members and two *Arabidopsis thaliana Esyt* members. Phylogenetic analysis of these gene products indicates a closer relationship between the NTMC2T2 and NTMC2T4 families than between the NTMC2T2 and NTMC2T3 families. The NTMC2T3, NTMC2T5, and NTMC2T6 groups appear unrelated to the other families at the gene structure level, and a greater degree of amino acid sequence similarity exists between the NTMC2T3 and NTMC2T6 families.

To characterise the structure of NTMC2s, the TM domain, synaptotagmin-like mitochondrial membrane protein (SMP) domain, and C2 domain were determined using several bioinformatics websites. Schematic representations of the six types of NTMC2 are shown in Fig. 1B. All NTMC2s contain at least one N-terminal TM domain, except for OsNTMC2T2.2 and OsNTMC2T4.2. We further found that NTMC2T4.1 and NTMC2T5 contain three and four TM domains, respectively. In addition, two C2 domains (C2A and C2B) were found in NTMC2T1, NTMC2T2, and NTMC2T3; however, only one C2 domain was found in NTMC2T4, NTMC2T5, and NTMC2T6. Interestingly, the SMP domain was also found in every NTMC2 protein. Multiple alignments of the C2A and C2B domains from NTMC2T1 to NTMC2T3, and the C2 domain from NTMC2T4 to NTMC2T6, were generated using the ClustalW2 algorithm and presented using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) (Robert and Gouet, 2014) (Fig. 1B), and showed the amino acid residues of the conserved calcium binding motif (loops 1 and 3) in these C2 domains. These results indicate that NTMC2 proteins have a conserved protein structure in *Arabidopsis* and rice.

3.2. Exon–intron structure analysis of OsNTMC2 genes

Analysis of the genome sequences of *OsNTMC2s* revealed that the lengths of the *OsNTMC2* genes range from 4.3 kilobases (*OsNTMC2T2.1* is 4365 bases) to more than 10 kilobases (*OsNTMC2T1.2*, *OsNTMC2T2.2*, and *OsNTMC2T4.1*). Exon–intron structure analysis was performed using GSDS. We found that the exon/intron structures of the *OsNTMC2* genes are nearly identical within the same group in the phylogenetic tree, but differ dramatically between groups (Fig. 2). All members of the *OsNTMC2T1* group had 12 exons and the intron phase was (0, 0, 0, 1, 0, 0, 0, 2, 0, 0, 0). *OsNTMC2T2.1* had 13 exons and the intron phase was (0, 0, 0, 1, 0, 0, 0, 2, 0, 0, 0, 0). Nine exons were found in *OsNTMC2T2.2*, which lacked the first four exons of *OsNTMC2T2.1*, and the intron phase was (0, 0, 0, 2, 0, 0, 0, 0). *OsNTMC2T3* and *OsNTMC2T6.1* also had 12 exons each and the intron phase was (0, 0, 0, 0, 1, 0, 0, 1, 1, 2, 0). Compared with *NTMC2T6.1*, *NTMC2T6.2* had 13 exons and the intron phase was (0, 0, 0, 0, 0, 0, 0, 0, 1, 1, 2, 0). Eleven exons were found in *NTMC2T4.1* and the corresponding intron phase was (0, 0, 0, 1, 0, 0, 0, 2, 0, 0). Compared with *NTMC2T4.1*, *NTMC2T4.2* had 12 exons; it had the first two additional exons and lacked the last exon. Its intron phase was (1, 0, 0, 0, 0, 1, 0, 0, 0, 2, 0). *OsNTMC2T5* was the only member of the type 5 group and had only six exons, with the intron phase (2, 1, 1, 0, 0, 1). These results suggest that *OsNTMC2T1*, *OsNTMC2T2.1*, *OsNTMC2T4.1*, *OsNTMC2T3*, and *OsNTMC2T6.1* separately present a high degree of gene structure similarity, which is in agreement with the phylogenetic tree analysis.

3.3. Expression patterns of OsNTMC2 genes in different rice tissues

The next-generation sequencing expression data of *OsNTMC2s* were extracted from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/). The RNA-Seq and TRAP RNA-Seq data were used to analyse the expression profiles of *OsNTMC2s* in organs during different developmental stages. The *OsNTMC2* FPKM values from leaves 20 days after germination, post-emergence inflorescence, pre-emergence inflorescence, panicles, anthers, pistils, seeds 5 days after pollination, seeds 10 days after pollination, embryos 25 days after pollination, endosperm 25 days after pollination, shoots, seedlings, and callus and those of panicles, seedlings, and callus from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/) were re-analysed and shown in a heat map with hierarchical clustering (Fig. 3A). Based on their expression values, 13 *OsNTMC2* genes were classified into two subgroups: *OsNTMC2T1.1*, *OsNTMC2T1.2*, *OsNTMC2T2.2*, *OsNTMC2T4.1*, and *OsNTMC2T5* were placed in one subgroup, as they were constitutively highly expressed in different tissues, and the other members were placed in another subgroup, and had relatively lesser expression in various tissues. However, all 13 *OsNTMC2* genes were expressed during at least one developmental stage or in at least on tissue. Microarray data from OsNTMC2s showed they also clustered into two subgroups; *OsNTMC2T1.5*, *OsNTMC2T3*, *OsNTMC2T4.1*, and *OsNTMC2T6.2* were placed in one subgroup and the other members were placed in another group (Fig. 3B). Using these two different methods to detect the expression of OsNTMC2s, we found that *OsNTMC2T4.1* is relatively highly expressed in various tissues during different developmental stages. We performed qRT-PCR to show that all *OsNTMC2s* are more highly expressed in roots 14 days after pollination, shoots, seedlings, and leaves 20 days after germination than in other adult tissues (Fig. 3A and C). *OsNTMC2T4.1* expression levels were 2739 times, 3206 times, and 6850 times that of *OsNTMC2T4.2*, which has the lowest expression level in this family, in young leaves, flowers, and seeds, respectively. *OsNTMC2T1.3*, *OsNTMC2T1.4*, and *OsNTMC2T4.2* had the lowest expression of the *OsNTMC2* family in nearly all tissues, except *OsNTMC2T4.2* in anthers (Fig. 3D). These results are consistent with the RNA-Seq and qRT-PCR data, and the diverse expression levels of OsNTMC2 family genes in different tissues suggest that they function in complex roles at all developmental stages in plants, and are expressed preferably in fast-growing young tissues.

3.4. Expression of OsNTMC2 genes in response to abiotic stresses

To determine the expression responses of *OsNTMC2s* to abiotic stress, the microarray data (GSE6901) of 7-day-old seedlings subjected to drought, salt, and cold stresses were analysed. All 13 *OsNTMC2* genes were detected in 7-day-old seedlings; however, only two genes were up-regulated. *OsNTMC2T6.1* was up-regulated 2.73-fold by drought stress compared with the control, whereas *OsNTMC2T6.2* was up-regulated 8.26-fold by drought stress and 3.09-fold by salt stress treatment compared with the control (Fig. 4). The expression levels of the other genes suggest that they are not stress responsive. These results indicate that the *OsNTMC2T6* genes may participate in abiotic stress signalling pathways and play important roles in response to these stresses.

4. Discussion

4.1. Phylogenetic analysis of the NTMC2 protein family in rice

NTMC2 proteins share domain architecture and amino acid sequence similarity and play roles in Ca^{2+}-mediated membrane fusion in eukaryotes through their calcium- and membrane-binding activities. In this study, six OsNTMC2 family groups with 13 members were

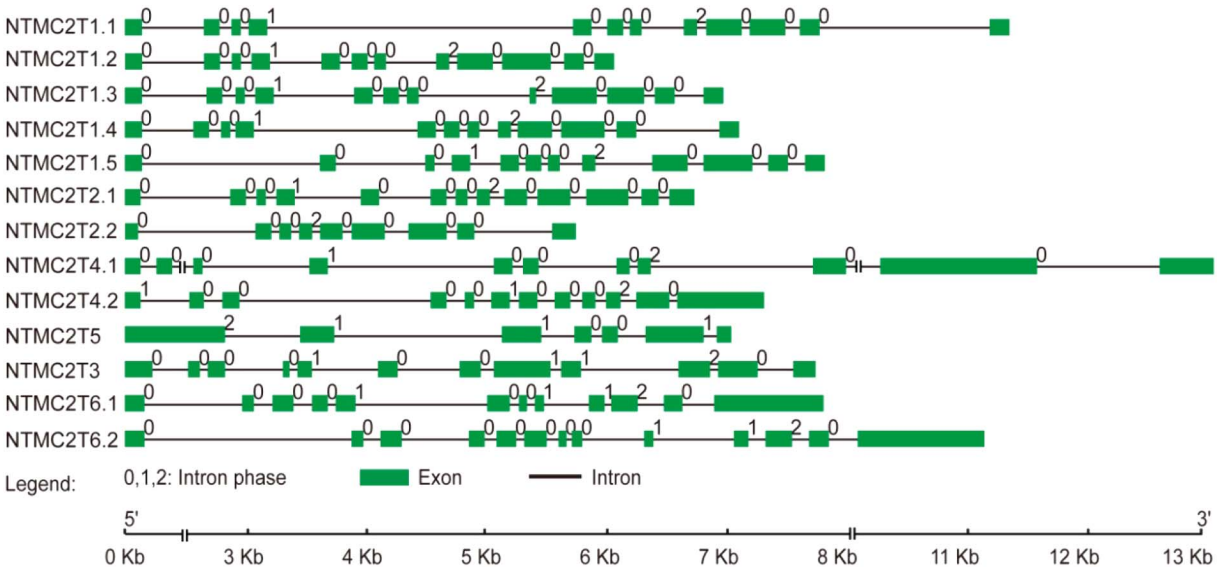


Fig. 2. Exon and intron organisation of corresponding *OsNTMC2s* genes.
Exon and intron organisation analysis was performed using sequences from the MSU Rice Genome Annotation Project Database. The exons and introns are represented by green boxes and black lines, respectively. The intron phases are marked 0, 1, and 2. This analysis was performed using GSDS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

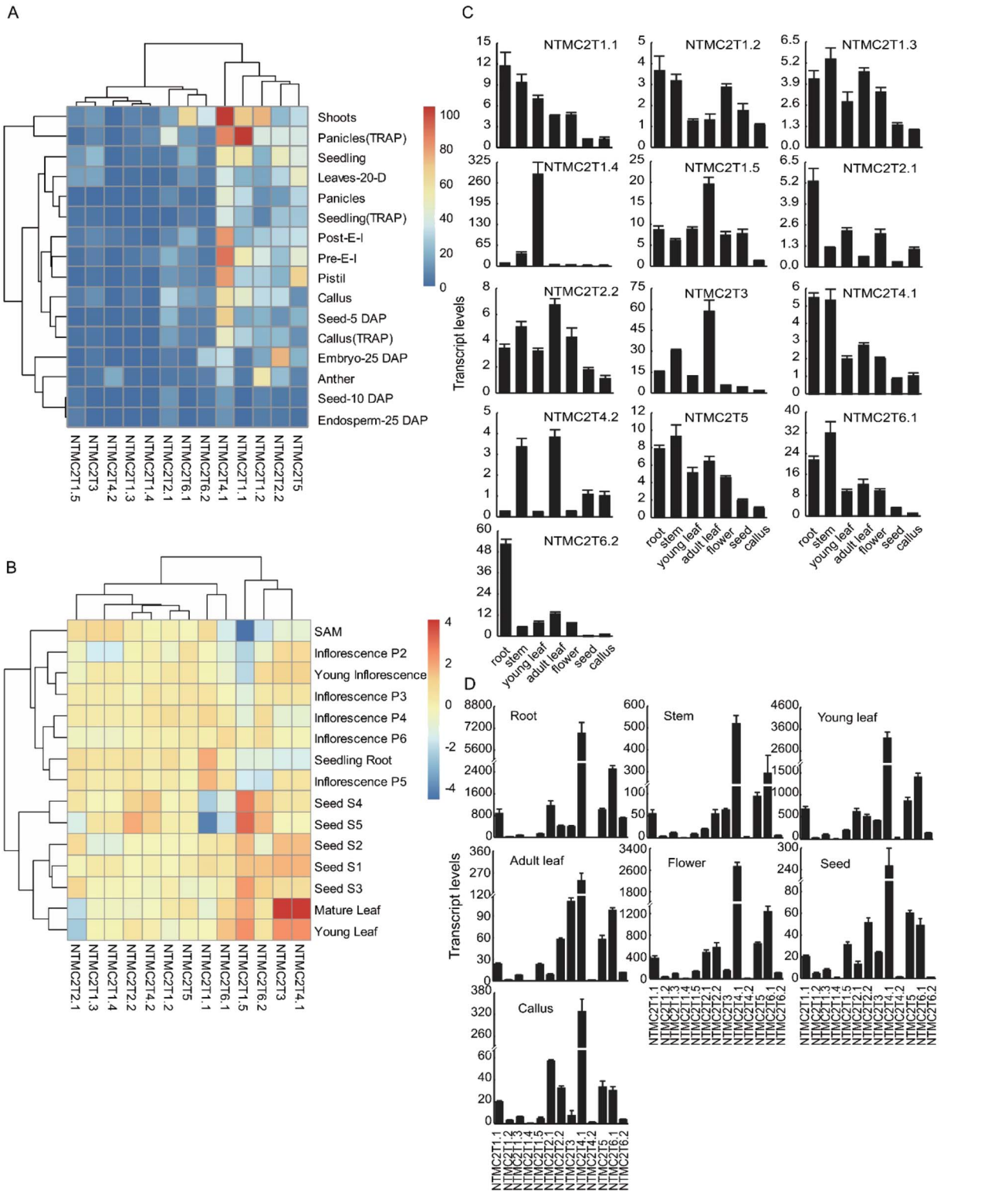
identified in the rice genome and possessed the highly conserved N-terminal TM domain, SMP domain, and C-terminal C2 domain. In addition to the high degree of homology between every pair of OsNTMC2 members, close phylogenetic relationships were found among the OsNTMC2T1, OsNTMC2T2, and OsNTMC2T4 groups. A previous study showed that gene evolution between NTMC2T2 and NTMC2T4 occurred by a gene fusion or fission event (Craxton, 2007). Here, we found that a deletion of the first four exons in *OsNTMC2T2.2* compared with *OsNTMC2T2.1* caused the deletion of the N-terminal TM and partial SMP domains in OsNTMC2T2.2. *OsNTMC2T4.2* has the first two additional exons and lacks the last exon compared with *OsNTMC2T4.1*, which causes a deletion of the N-terminal TM and two C-terminal TM domains in OsNTMC2T4.2. These results indicate that a deletion or replication event occurred in the genomes of *OsNTMC2T2.2* and *OsNTMC2T4.2*. Similar to OsNTMC2T1 and OsNTMC2T2, OsNTMC2T3 has two C2 domains, but it has greater degrees of gene structure and amino acid sequence similarity to the OsNTMC2T6 family, which validates the nomenclature suggested by our phylogenetic analysis. The main structural characteristics of the gene and protein sequences of OsNTMC2 members were formed prior to rice speciation.

Every OsNTMC2 had high sequence homology to the DUF 2404 (SMP) domain in a search of the NCBI database (https://www.ncbi.nlm.nih.gov/) using the Conserved Domain Search Service. This domain was identified and named SMP in 2006 (Lee and Hong, 2006) and is the variable linker region between the N-terminus and the first C2 domain. Previous studies showed that the SMP region, combined with the N-terminal TM domain, may be sufficient for localisation to the ER (Endoplasmic reticulum) membrane, and the tandem C2 domains require the SMP domain to interact with the plasma membrane (Yamazaki et al., 2010). The TM-SMP-C2A fragment of AtSyt1 localises to the ER in the tobacco protoplast system (Lewis and Lazarowitz, 2010). We conclude that the SMP domain is critical for ER localisation of these proteins in eukaryotes. The OsNTMC2 C2 domains were also analysed. The C2A and C2B domains were found in NTMC2T1, NTMC2T2, and NTMC2T3. NTMC2T4, NTMC2T5, and NTMC2T6 each have only one C2 domain. From the amino acid sequence alignment results of C2A, C2B, and the C2 domains from OsNTMC2s, we found that the C2 domain in NTMC2T4, NTMC2T5, and NTMC2T6 is more similar to the C2A domain than to the C2B domain in NTMC2T1, NTMC2T2, and NTMC2T3, which is in agreement with previous results that the C2A domain, but not the C2B domain, Ca^{2+}-dependently binds lipids (Wang et al., 2015).

4.2. Expression patterns of OsNTMC2 genes in rice

In this study, RNA-Seq data from *OsNTMC2* genes in different tissues were analysed to reveal their expression patterns. The results showed that all 13 *OsNTMC2* genes were expressed in at least one development stage or one tissue, and were preferably expressed in fast-growing young vegetative organs, except *OsNTMC2T4.2*, which is specifically expressed in the anther. We also found that *OsNTMC2T4.1* has the greatest expression of the *OsNTMC2* family genes in all tissues. These results were further confirmed by qRT-PCR experiments. qRT-PCR results showed that *OsNTMC2T4.1* expression was 2739-fold, 3206-fold, and 6850-fold higher compared with *OsNTMC2T4.2*, which had the lowest expression levels for this family, in young leaves, flowers, and seeds, respectively. We also found that the expression level of *OsNTMC2T6*, but not other *OsNTMC2* members, was increased under drought and salt stress in the microarray data from 7-day-old rice seedlings. OsNTMC2T6 may participate in abiotic stress–mediated membrane repair pathways in rice. A previous study showed that the *NTMC2T1.2* gene was highly upregulated by ABA (abscisic acid) and drought stress in *Physcomitrella* (Cuming et al., 2007). AtNTMC2T1.1 was identified as a required participant in plasma membrane repair and as a regulator of endocytosis. Membrane fusion in the tip growth of vegetative organs has also been discussed (Rounds and Bezanilla, 2013). In addition, AtNTMC2T1.2 (also called Syt2) is expressed specifically in the stamen (Wang et al., 2008), participates in pollen germination and tube growth, and is delivered to the plasma membrane via conventional secretion (Wang et al., 2015). We found that *OsNTMC2s* were expressed preferably in shoots, leaves 20 days after germination, seedlings, and roots, which requires tip growth and membrane fusion. However, *OsNTMC2T4.2* was expressed specifically in the anther, suggesting that it plays a similar role to AtNTMC2T1.2 in *Arabidopsis* pollen development.

In summary, the complex gene structure and varied expression patterns of OsNTMC2s suggest that each member of the *OsNTMC2* family plays a distinct role in growth and development and in responses to diverse abiotic stresses. These results provide further data for the study of the physiological function of OsNTMC2s as calcium- and membrane-binding proteins in rice.



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Competing interests

The authors declare that they have no competing interest.

Authors' contributions

RH, JZ, YW, SH and HZ conceived and designed the study. RH, JL,

Fig. 3. Expression patterns of OsNTMC2 family genes in different tissues at various developmental stages.
(A) Expression profiles of Os*NTMC2* genes from RNA-Seq data obtained during different vegetative and reproductive developmental stages. A heat map representing hierarchical clustering of the FPKM values of *OsNTMC2* genes in various developmental stages was generated (Leaves-20-D; Post-E-I; Pre-E-I; Panicles; Anthers; Pistils; Seeds-5 DAP; Seeds-10 DAP; Embryos-25 DAP; Endosperm-25 DAP; Shoots; Seedlings; Callus; Panicles (TRAP); Callus (TRAP); Seedlings (TRAP); D: day; E-I: emergence inflorescence; TRAP: TRAP-Seq FPKM value; DAP: days after pollination). The heat map was generated using R x 64 v. 3.2.1 with the hierarchical clustering method. The colour scale (representing the FPKM values) is shown on the right.
(B) Expression profiles of *OsNTMC2* genes from genome-wide microarray data of GSE6893 during different vegetative and reproductive developmental stages. A heat map representing hierarchical clustering of average log signal values of OsNTMC2 genes in various developmental stages was generated (YR, roots from 7-day-old seedlings; ML, mature leaf; YL, leaves from 7-day-old seedlings, different stages of panicle development: SAM, up to 0.5 mm; P1, 0–3 cm; P2, 3–5 cm; P3, 5–10 cm; P4, 10–15 cm; P5, 15–22 cm; P6, 22–30 cm, and different stages of seed development: S1, 0–2 dap; S2, 3–4 dap; S3, 5–10 dap; S4, 11–20 dap; S5, 21–29 dap). A colour scale bar (representing average log signal values) is shown on the right.
(C) Relative quantification was determined by real-time RT-PCR analysis (with rice ubiquitin used as an internal control) of the expression levels of 13 *OsNTMC2*s in roots, stems, young leaves, adult leaves, flowers, seeds, and callus of the rice cultivar Zhonghua 15. The expression level of each gene in the callus was 1.0. Bars show the mean relative fold-change values from three biological replicates. Standard errors of the biological replicates are shown as error bars.
(D) Expression levels of different *OsNTMC2* members in the same tissue. Relative quantification was performed by real-time RT-PCR analysis (with rice ubiquitin used as the internal control) of the expression level of each member of the NTMC2 family in roots, stems, young leaves, adult leaves, flowers, seeds, and callus of the rice cultivar Zhonghua 15. The expression level of *OsNTMC2T4.2* was 1. Bars show the means of relative fold-change values from three biological replicates. Standard errors of the biological replicates are shown as error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

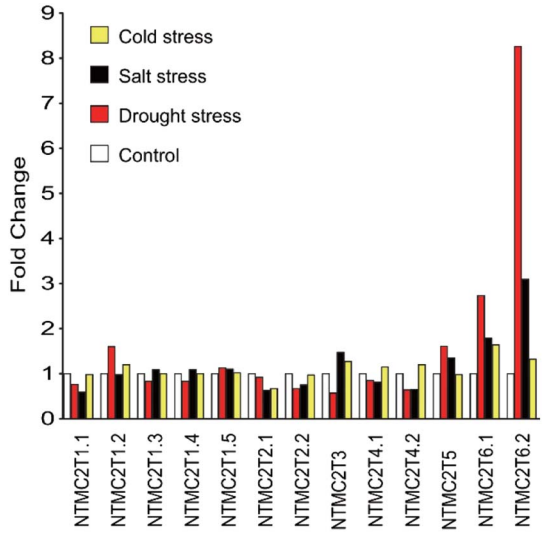

Fig. 4. Expression of *OsNTMC2* gene responses to different abiotic stresses.
A histogram showing the fold change of *OsNTMC2* genes under control, drought stress, salt stress, and cold stress conditions. The expression data were re-analysed from the genome-wide microarray data of GSE6901.

HZ and SH conducted the experiments and analysed the data. RH and SH wrote the manuscript. RH, JZ, SH and HZ revised the manuscript. All authors have read and approved the final version of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2017.09.046.

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