Overexpression of a thylakoid membrane protein gene OsTMP14 improves indica rice cold tolerance

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ABSTRACT
Cold has a major impact on rice, so rice can only grow in specific climate zones. TMP14 (thylakoid membrane protein of 14 kDa) is a nuclear genome-encoded chloroplast thylakoid membrane protein, which is involved in a wide range of abiotic stress responses. However, the knowledge of TMP14 roles in response to adverse stimuli is still very limited in rice. Based on the results of expression profiles, OsTMP14 (GenBank accession: BAF27084.1) was highly induced in both leaf and panicle at all the developmental stages of rice analyses under cold and drought stresses. Sequence analyses of OsTMP14 putative promoter regions identified nine cis-elements related to stress responses. Furthermore, transgenic rice plants with overexpression of OsTMP14 showed more tolerance to cold stress. Taken together, these results indicate that OsTMP14 is involved in stress tolerance in rice, which is useful in developing transgenic rice with enhancing tolerance to cold stress.

Introduction
Rice, which is both a model plant and an extremely important crop feeding more than one-third of the world’s population, evolved in subtropical and tropical areas and is sensitive to cold stress. Asian cultivated rice (Oryza sativa) consists of two major subspecies, japonica (O. sativa ssp. japonica) and indica (O. sativa ssp. indica). It was acclimatized from O. rufipogon and O. nivara [1,2]. Compared with temperate japonica, which are planted in regions with lower average temperatures (as typical japonica cultivars), indica cultivars have weaker cold tolerance. Cold is a major environmental stress which has negative impacts on plant growth and development as well as yield potential. Plants have developed the ability to respond to cold stress on a molecular, biochemical and physiological level, enabling them to survive in the stressful conditions [3]. The molecular mechanisms underlying the domestication of higher plants to various abiotic stresses have attracted a lot of interest, as sub- jected to cold stress result in the seriously loss of crop yield in most parts of the world [4,5]. A number of cold stress-induced genes have been discovered recently using molecular and genetic approaches as microarray experiments [6,7]. The products of these special cold stress-induced genes are generally considered to enhance cold stress tolerance and modulate gene expression via signalling pathways [8,9]. Plants respond to cold stress challenges in part through altering their gene expression levels, which finally leads to various adaptive responses at cell and whole-plant level [10].

Photosystem I (PSI) is a super membrane protein complex which catalyzes the first step of oxygenic photosynthesis driven by green algae, cyanobacteria, and higher plants. Structurally, higher plant PSI consists of 5 different antenna proteins, 15 core subunits and 2 membrane complexes [11]. The roles of the three plastome-encoded PSI subunits, PsaA, PsaB and PsaC, which are indispensable for PSI assembly and biological function, because all redox-active cofactors are bound by them. Lack of these subunits, activated PSI cannot accumulate in photosynthetic eukaryotes [12]. Previous reports showed that the core proteins of PSI were related to stresses. For example, the overexpression of OsCEST (AK068219) improved tolerance to salt, drought, and heat stresses and the overexpression of homologous gene AtCEST (At5g44650) from Arabidopsis also had a high salt tolerance [13]. TMP4 as a thylakoid membrane protein in PSI, encoded by a nuclear gene, which is phosphorylated in vivo and has a role in LHCl stability [11,14–16]. Homologues of TMP14 were predicted to be present in cyanobacteria and higher plants but not in green algae. In the six kinds...
of higher plants, *Arabidopsis thaliana*, *Populus tremula*, *Salix viminalis*, *Mesembryanthemum crystallinum*, *Zea mays*, and *O. sativa* as well as in cyanobacteria, all the proteins encoded by homologous genes of PSIP contain two transmembrane regions, but the computer programs could not accurately calculate the transmembrane regions of the cyanobacterial proteins [16]. All of the plant TMP14 genes have well defined signalling peptides through the predicted high probability for chloroplast-targeting [17]. The results of topology prediction from the plant proteins revealed that the N-termini of TMP14 presents in the stromal side of thylakoid membrane, which is consistent with the phosphorylation of TMP14 at the chloroplast matrix side of the membrane [16]. Finally, it revealed that the phosphorylation site of TMP14 in Arabidopsis is one of the two threonine on the N-termini of the polypeptides. However, the functional analyses and molecular mechanisms of stress-response from plant TMP14s are not yet understood.

In the present paper, GeneChip Rice Genome Array (Affymetrix) was used to screen a stress tolerance candidate gene *OsTMP14* from cultivar Pei’ai 64S. The *OsTMP14* gene encoding thylakoid membrane protein of 14 kDa was highly induced by cold stress and drought stress in the leaves of booting and tassels of booting stages, which is relevant to stress resistance. To further explore the mechanism of *OsTMP14* involvement in stress tolerance, transgenic rice with overexpression of *OsTMP14* was generated. Transgenic rice plants showed improved cold tolerance.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of Pei’ai 64S (*O. sativa ssp. indica*) were suspended in a sterile solution of 0.1% HgCl₂ for 10 min, washed five times using distilled water, immersed for 3 days at 25 °C (water was changed once a day), then germinated at 37 °C for 3 days in distilled water. They were broadcasted in batches in net basin of Rice Research Institute of Sichuan Agricultural University. In this study, plants were divided into one control and three treatment groups. The control group was maintained under normal growth conditions and the treatment groups were exposed to heat, cold and drought stresses. At five-leaf stage, parts of them were harvested as the test materials in the seedling stage. The rest were transplanted to other pots until pots contained five plants per pot, and were harvested as test materials in flowering and booting stage.

Cultivar 93-11 (*O. sativa ssp. indica*) plants were germinated and grown under white fluorescent light (500 μmol m⁻² s⁻¹, 12 h light periods) at 30 °C in a greenhouse. Seeds were sterilized with 75% ethanol for 5 min, bleached with 2% sodium hypochlorite for 30 min. Finally, seeds were rinsed with sterile water, and then incubated for 2 d at 37 °C under dark conditions. Plants were grown on 1/2 Murashige and Skoog (MS) medium with 0.8% (w/v) agar and 1.2% (w/v) sucrose in a growth room maintained at 28 °C and 65% relative humidity under a physiological photoperiod of 12 h light and 12 h darkness.

**Rice cold, heat and drought treatment for microarray analysis**

Under the cold stress test, we placed the rice seedlings of five-leaf stage into climate incubator, PGC15.5 (Percival Scientific, Perry, IA, United States) with 4 °C for 12 h and booting as well as heading stage with 12 °C for 16 h. Under the heat stress test, the treatment group was put into PGC15.5 for 2 h under 45 °C, while the control group was put into PGC15.5 under 28 °C. Under the drought stress test, the watering was poured away from the basin until the treatment group dried out and the control group was put in the dry shed with water in pots, then the leaves were collected when they started curling after 16 h. All groups were under dark conditions.

**Microarray data analysis**

The process was performed according to procedures previously described by Chen et al. [18]. In brief, according to Affymetrix expression microarray experiments manual provided by GeneTech Biotechnology Limited Company, Shanghai.

**Motif identification**

We identified the protein motifs of TMP14 genes by MEME ([http://meme.sdsc.edu/meme/meme.html](http://meme.sdsc.edu/meme/meme.html)), here the motif sites set at 20, motif length 3–300, and the largest number of motif sites to find was defined as 20. Then the results were manually adjusted. Moreover, we characterized the two motifs by DNAMAN ([http://www.lynnnon.com/](http://www.lynnnon.com/)).

**cDNA cloning**

Full-length of *OsTMP14* cDNA was amplified by high fidelity HiFi taq DNA polymerase (Transgen, Beijing, China). Special primers were designed by the software primer-premier 5.0 after searching homology cDNA sequence, which were *OsTMP14*-F: 5’-GGATCCGTACAGTTAGTATGACACCGACCAGT-3’, *OsTMP14*-R: 5’-GAATTCAATCCAGTAGCAGCCTCCCTT-3’. The PCR cycles were
performed as follows: an initial denaturation for 5 min at 95 °C, 30 amplification cycles, [30 s at 95 °C (denaturation), 30 s at 59 °C (annealing), and 1 min at 72 °C (polymerization)], followed by a final elongation for 7 min at 72 °C. All the PCR fragments were purified by Gel Extraction Mini Kit (Biomed, Shanghai, China), the amplified fragment was ligated into vector pMD18-T (TaKaRa, Dalian, China), then cloned into Escherichia coli strain Top10 (Transgen, Beijing, China). The positive transformants were screened by using kanamycin selection. And restriction enzymes BamH I and EcoR I (TaKaRa, Dalian, China) were used for double cuts identification. The positive clone materials by screening were sequenced by Invitrogen (Biotechnology Limited Company, Shanghai, China).

Promoter analysis

About 1500 bp ahead of the translation initiation codon (ATG) in DNA sequences of rice OsTMP14 gene were obtained from the GRAMENE database (http://www.gramene.org/). The promoter of OsTMP14 was analyzed by Plant-CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and the results were verified by the PLACE database (https://www.ncbi.nlm.nih.gov/pubmed/9847208).

Phylogenetic analysis

All amino acid alignments of the identified TMP14 genes were conducted by ClustalW (www.ebi.ac.uk/clustalw/). The phylogenetic tree was generated by the Neighbour-Joining method and visualized using the same software MEGA (http://www.megasoftware.net/) [19]. We used 1000 replicates, p-distance method and pairwise deletion gaps data treatment for bootstrap analysis.

Construction of the expression vector and rice transformation

To construct the overexpression of plant transformation vector, OsTMP14 was ligated into the binary expression vector D-163+1300 (derived from pJIT163 and pCAMBIA1300), in which it transferred gene expression under the control of CaMV 35S promoter. For rice transformation, D-163+1300-OsTMP14 was introduced into Agrobacterium tumefaciens EHA105 cells and then transformed into rice 93-11 (O. sativa L. ssp. indica). Transformed seeds were selected on 1/2 MS medium with 50 mg/L hygromycin. The expression levels of OsTMP14 in different transgenic lines were checked by semiquantitative reverse transcription-PCR (RT-PCR).

Analysis of transgenic plants for cold stress tolerance

The positive plants of OsMTP14-OE T0 lines were used in cold stress assays. In order to evaluate their tolerance to cold stress, the seedlings at the three-leaf stage of the WT (Pei’ai 93-11, O. sativa ssp. indica) and transgenic lines were incubated at natural low temperature from September to November (2011, Wenjiang, Chengdu). Then we observed the colour of rice seedling and recorded the survival rates.

Figure 1. Expression pattern of PSI genes in genome coding core proteins during stresses under different tissue and development for microarray experiment. (A and B) Hierarchical clustering of the genes showing low levels of expression in the stresses; (C) the changed fold of OsTMP14 (psaP) is shown. The vertical lines on the right side of the map show the genes. Dashed line expressed more than two-fold in any one stress. 1: seedling stage; 2: booting stage; 3: heading and flowering stage; L: leaf; S: spike; CK: control; CS: cold stress; HS: heat stress; DS: drought stress; SS: salt stress. The colour bar representing log2 signal values is shown.
Results and discussion

Expression analysis of OsTMP14 gene during abiotic stresses

According to the results of GeneChip, among the nine genes encoding the core protein of PSI, the expression pattern of psaP (OsTMP14) was different from that of the other eight genes (Figure 1(A,B)). The expression level of psaP was only very high in the leaves of seedling stage (1L) and low in the control group under other normal conditions. PsaP in different growth stages can be induced more than two-fold by low temperature (Figure 1(C)), while 7-day leaves had a little change with less than two-fold (Figure 1(B)). PsaP was upregulated by drought induction in booting (2L) and tassel of booting (2S) stages, while it was downregulated by high temperature in heading stage (3S) (Figure 1(C)). PsaP in other periods of stress induction has changed but not considerably. Only psaH and psaO were upregulated by drought induced in the booting stage, while other genes were downregulated under stress in different developmental stages. The expression levels of the nine genes in the period of 7-day leaves have little changes under salt stress.

Cloning and sequence analysis of OsTMP14

To fully analyze OsTMP14, we designed the specific primers using the software primer-premier 5.0 based on the conserved region after GenBank was searched, and then the cDNA sequence of OsTMP14 containing the complete ORF was cloned by RT-PCR from Pei’ai rice 64S. OsTMP14, which is located in chromosome 10, contains five introns (Figure 2). Sequence analysis showed that OsTMP14 cDNA encodes a protein of 172 amino acid residues with theoretical M.W. about 17.95 kDa, pl about 7.85 and shared 100% identity to the corresponding sequence of Nipponbare (GenBank: AK066849, range: 20,821,977–20,823,699). In order to get the protein structure information of OsTMP14, protein prediction online software InterProScan and ScanViewto were used to deduce its secondary structure (Figure 3). There were one SNP, one antifreeze-1 domain, one LppLPgLASHgQPrvaS, one His47 and one DUF4308 superfamily domain with aminoacyl-tRNA-ligase II-2, and seven potential phosphorylation sites: one Ser, two Tyr and four Thr. The possible promoter region (range: 10:20,820,237-10:20,822,276) contains many different cis-acting elements, there were nine cis-acting elements related to stress: three ABRE (ABA-responsive element), one GC-motif (anaerobic-related cis-elements), two TGACG-motif (JA-responsive element), one CGTCA-motif (JA-responsive element), one ATGCAAAT-motif (TGACGTCA motif-related cis-elements) and one O2-site (kinetin regulatory element) (Figure 4). The existence of these stress-related cis-elements showed that with the promoter region of OsTMP14 responses to various kinds of stress signals, the expression of OsTMP14 is regulated by several stress factors.

Phylogenetic analysis of OsMTP14

The database search and analysis with BLASTp through the NCBI website revealed that the deduced amino acid sequence of OsMTP14 has complete identity (100%) with protein NP_001065170 encoded by AK066849 from Nipponbare. BLASTp showed that OsMTP14 has the highest homology with MTP14 from other plant species. The percentages of identity were from 52% to 99% compared to protein-coding sequences from other genes. The length of protein sequences is between 146 aa and 174 aa, and the similarity of C-terminal sequence is large. Research showed that the N-terminal region of TMP14 protein in most plants has about 30 amino acid sequences as the signal peptide region and the C-terminal region contains two transmembrane structures (Figure 5).
OsMTP14 with several corresponding proteins from rice and other plant species showed that MTP14 domains were highly conserved in these proteins, suggesting that they have the same biological functions, probably involved in stress response. According to the NCBI database, it was found that the TMP14 gene copy number predicted by different species was different. The soybean (Glycine max) TMP14 gene has three copies, Ricinus communis, Vitis vinifera and Brachypodium distachyon have two copies, O. sativa, Z. mays, A. thaliana, Medicago truncatula and Hordeum vulgare subsp. vulgare have only one predicted TMP14 gene.

The full-length amino acid sequences of OsMTP14, several candidate rice MTP gene family members and the similar genes in other species were used to construct an unrooted phylogenetic tree (Figure 6). The phylogenetic tree has three branches, in the aligned sequences, OsTMP14 had the closest genetic relationship with

Figure 4. Sequences of OsTMP14 candidate cis-elements in the putative promotor region. The transcriptional start site C is denoted +1, ABRE (ABA-responsive element), GC-motif (anaerobic-related cis-elements), TGAGC-motif (JA-responsive element), CGTCA-motif (JA-responsive element), ATGCAAAT-motif (TGAGCCTA motif-related cis-elements) and O2-site (Kinetin regulatory element). ‘…’ represents bases not printed.

Figure 5. Alignment of the deduced protein sequences of OsTMP14 with other similar sequences in plants. The numbers on the right indicate the positions of the last amino acids on the line. The signal peptide domain is underlined with black line; transmembrane regions are boxed; ‘...’ for no consensus; conserved aa residues are shaded.
Overexpression of OsMTP14 increased tolerance to cold stress in rice

To further characterize the biological function of the OsMTP14 gene in rice, transgenic rice plants overexpression of OsMTP14 were generated. To determine the performance of transgenic rice under cold conditions, the seedlings at the three-leaf stage of the wild-type 93-11 and transgenic lines were incubated at natural low temperature (2011, Wenjiang, Chengdu) from September to November. The results showed that the leaves of T0 transgenic rice plants turned yellow slower comparing with wild-type 93-11. Furthermore, the transgenic plants leaves remained green, while the wild-type 93-11 mostly turned yellow (Figure 7). Taken together, the results indicated that overexpression of OsMTP14 in rice enhanced the cold tolerance in transgenic rice plants. Previous studies suggested that COLD1 could act as a cold sensor to perceive cold stimuli and determines cold tolerance in rice [20–22]. OsMTP14 may play a function downstream of gene COLD1.

When plants are subjected to environmental stresses, the proteins of chloroplast play an important role in terms of resilience. Forty percent of the cold-response proteins may be located in the chloroplast, and 90% of the chloroplast proteins are encoded by the nuclear genomes and then transported to the chloroplast [15]. The results of microarray showed that the expression of several core proteins of the PSI encoded by nuclear genes is different in stress conditions (Figure 1), only psaH and psaO were upregulated by drought induced in the booting stage, while other genes were downregulated under stress in different developmental stages. These types of core protein of the PSI play a key role in the photosynthesis of chloroplast, psaH and psaO may be involved in drought-response in rice [14,15,23]. TMP14 is a thylakoid membrane protein encoded by the nuclear gene (psaP) in the chloroplast, OsMTP14 gene is

**Figure 6.** Phylogenetic tree of the protein sequences of OsTMP14 and other similar sequences. Dendrogram branches are labelled with percentage of 1000 iterations supporting each branch. Bootstrap values are shown in the nodes of the tree. EAY79344.1 (Oryza sativa indica Group), EAZ16812.1 (Oryza sativa japonica Group, uncharacterized protein), BAK01756.1 (Hordeum vulgare subsp. vulgare), XP_003574260.1 (Brachypodium distachyon), NP_001130557.1 (Zea mays, uncharacterized protein), XP_002266107.1 (Vitis vinifera), XP_003548086.1 (Glycine max), XP_003626612.1 (Medicago truncatula), XP_003521827.1 (Glycine max), NP_566086.1 (Arabidopsis thaliana), NP_001150124.1 (Zea mays), XP_003563146.1 (Brachypodium distachyon), XP_002516019.1 (Ricinus communis), XP_003521828.1 (Glycine max), XP_002284539.1 (Vitis vinifera) and XP_002511952.1 (Ricinus communis).

EAY79344.1 (indica) and EAY79344.1 (japonica, undefined functional protein), and OsTMP14 is also relatively close to BAK01756.1 and XP_003574260.1. The results of sequence phylogenetic tree analysis showed that OsTMP14 has a close relationship with protein sequences except R. communis XP_002511952.1 and XP_002516019.1, V. vinifera XP_002284539.1.

**Figure 7.** Performances of transgenic rice and wild-type 93-11 under cold conditions. Transgenic rice survived better than wild-type plants under cold stress.
significantly upregulated in response to hypothermia and drought at different growth stages and different tissues and organs, while the high temperature induced significant downregulation of the OsTMP14 gene. This suggested that OsTMP14 is involved in cold and drought-response in rice. Moreover, the expression of OsTMP14 can be affected by other core proteins expression of PSI, for example, when the expression of the PSI-G decreased 40%, the content of TMP14 dropped more, so the low temperature and drought stress-response of OsTMP14 is related to other PSI core proteins [24].

The possible promoter region contains many cis-acting elements which are associated with stress-response (Figure 4). It was previously reported that the ABA-responsive element ABRE was related to low temperature stress, moreover, CGTCA-motif, GC-motif, TGACG-motif, O$_2$-site and ATGCAAAT-motif were related to various stresses [25-28]. This indicated that OsTMP14 may be involved in the tolerant adaptation processes of rice. Furthermore, there are four cis-acting elements (one ATGCAAAT-motif, two ABRE and one TGACG-motif) located downstream of the transcription initiation site which is related to the process of TMP14 transcription in the nucleus and then play a role in the chloroplast [26,28,29]. According to the analysis results of the predicted protein sequence encoded by OsTMP14 (Figure 3), the proteins encoded by OsTMP14 contain a domain of antifreeze protein-1-like and have similar domains compared with the structure of antifreeze protein-1-like of fishes from the polar regions [30]. This revealed that these proteins may play a role in hypothermia response; moreover, the results of gene chip analysis also showed this potential function. Meanwhile, these proteins contain a proteolytic enzyme domain of serine, OsTMP14 is phosphorylated in vivo, while the phosphorylation site is different from Thr65 of Arabidopsis, perhaps is Ser121, Thr104, Tyr 100 or Tyr166, which needs further investigation [11].

TMP14 gene has been sequenced in many plants, some species have multiple gene copies, for example, TMP14 of G. max has three gene copies, R. communis, V. vinifera and B. distachyon have two copies. However, some TMP14 proteins of plants have smaller variation and closer genetic relationship in the evolutionary process, for example, XP_002516019.1 and XP_002511952.1 protein of R. communis in different branches, XP_002516019.1 protein of R. communis and XP_002284539.1 protein of V. vinifera have closer relationship, NP_001130557.1 of Z. mays and OsTMP14 have closer genetic relationship. Another unknown function protein (EAZ16812.1) of rice has 83% homology with OsTMP14 (Figure 6). Understanding the evolution of TMP14 protein provides a clue to further study its function. The two transmembrane regions and nearby amino acid sequences of TMP14 protein are highly conserved, this region is an unknown functional domain of DUF4308 superfamily which contains about 90 amino acids, wherein the II-2 region of aminoacyl-tRNA-ligase is also highly conserved (Figure 3). Furthermore, it has been previously reported that aminoacyl-tRNA-ligase in microorganisms was related to the function of temperature-sensitive mutant (E. coli K-12) and cis-acting elements T-box [31]. Taken together, this region of MP14 protein may be involved in stress response.

So far, there has been some research on location and function of other core proteins of PSI from plants, while there is limited understanding of the function of TMP14, Jackson reported TMP14 had an important role in antihistidin (Diuraphis noxia) in wheat [14,24,32,33]. In addition, the mechanisms of the proteins encoded by OsTMP14 have not been clearly elucidated, so we constructed the overexpression of OsTMP14, obtained positive T0 transgenic rice plants and made a preliminary observation of cold stress. The results indicated the overexpression of OsMTP14 in rice enhanced the cold tolerance in transgenic rice plants (Figure 4). To further study the function of OsMTP14, RNAi and GFP, as well as promoter vector of OsMTP14, would be constructed for related experiments, and then the phenotypic identification of T2 homozygous lines under various abiotic stresses would be performed.

**Conclusions**

This work describes the overexpression of a thylakoid membrane protein gene OsTMP14 improves 93-11(O. sativa ssp. indica) cold tolerance. Compared with temperate japonica, which are grown in regions with lower average temperatures, indica cultivars have weaker chilling tolerance. Based on the results of expression profiles, OsTMP14 (thylakoid membrane protein of 14 kDa), which was highly induced in both leaf and panicle at all the developmental stages of rice analyses, responded to cold and drought stresses. Nine cis-elements related to stress responses were found in the predicted promoter region, which further certified that OsTMP14 is related to stress tolerance. Furthermore, transgenic rice plants with overexpression of OsTMP14 showed more tolerant to cold stress and displayed higher survival rates. Taken together, OsTMP14 plays a positive role in plants’ cold tolerance.

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Disclosure statement

The authors declare that they have no conflict of interest.

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