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Identification and characterization of a novel abiotic stress responsive *ATPase* gene from rice

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Abstract

Adenosine triphosphatases (*ATPases*) belonging to the AAA protein family (*ATPases* Associated with various cellular Activities) are involved in a wide range of abiotic stress. *OsATPase*, is one of the gene among these stress genes. However, the knowledge about their roles in developmental processes and response to various stimuli are still very limited in rice. In order to discover new stress tolerance genes in rice (*Oryza sativa L.*), expression profiles were obtained for leaf and panicle tissues at seedling, booting and heading stages of *indica* cultivar Pei'ai 64S plants under cold, drought or heat stresses using the GeneChip Rice Genome Array (Affymetrix) representing 51, 279 transcripts from japonica and *indica* rice. *OsATPase* was highly expressed in leaves and panicles and in response to cold stress in seedlings and booting stage. Real-time quantitative PCR analysis showed that the result was almost consensus with GeneChip Rice Genome Array, suggesting that *OsATPase* is a multiple stress responsive gene in rice. In order to study its function in stress tolerance, we cloned the cDNA of the gene through amplification by RT-PCR. Sequence analysis showed that the cDNA encodes a protein of 528 amino acid residues with M.W.≈60kD and pI≈7.8. Analysis of the putative promoter region for candidate *cis*-regulatory elements using Plant CARE software identified some *cis*-elements related to stress responses. Based on the earlier mentioned analysis and results obtained, we propose that *OsATPase* is a novel candidate gene involved in stress tolerance in rice.

Key words: *Oryza sativa L.*; microarray, *ATPase*; abiotic stress; cold and salt stress.
Abbreviations: *Os_Oryza sativa L*; AAA_*ATPases* Associated with various cellular Activities; *ATPase*_Adenosine triphosphatase; M.W_molecular weight.

Introduction

Rice (*Oryza sativa L.*) is an important food crop serves as a major source of carbohydrates for more than one-third of the world's population and it is widely cultivated in arable land worldwide (Jain et al. 2007). Various adverse environmental stresses induce the expression of a variety of genes in many plant species (Bartels and Sunkar 2005;Shinozaki et al. 2003;Xiong et al. 2002). Abiotic stresses profoundly influence the overall growth and development of rice, including reproductive success and crop yields. Therefore, rice has evolved versatile strategies to rapidly sense environmental fluctuations and activate adaptive responses under extremes environment. Low temperature, drought and high salinity are common stress conditions that adversely affect plant growth and crop production (Duan et al. 2009; Jiang et al. 2011; Tan et al. 2013; Xiong et al. 2002). To understand the mechanism by which rice perceives environmental signals and transmit the signals to cellular machinery to activate adaptive responses and isolate stress-related genes for developing stress-tolerant rice which is significant for ensuring stable and high yield, raising the utilization efficiency of low-yield fields, expanding rice planting area, we investigated the gene expression levels of rice in different stresses. Numerous stress-induced genes have been identified using microarray experiments (Kreps et al. 2002; Seki et al. 2002). The products of these genes are thought to promote stress tolerance and regulate gene expression through signal transduction pathways (Shinozaki et al. 2003; Xiong et al. 2002). Previous reports indicate that significant portion of the plant genome participate in the plant response and acclimation to drought, cold and heat stresses (Shinozaki and Yamaguchi-Shinozaki 2000; Shinozaki et al. 2003; Xiong et al. 2002; Zhu 2001). Change in the expression of a single gene can enhance the ability of plants to withstand drought and salt stresses (Garg et al. 2002; Haake et al. 2002; Kasuga et al. 1999; Xu et al. 1996; Zhang et al. 2004). Adenosine triphosphatases (*ATPases*) belongs to the AAA protein family (*ATPases* Associated with various cellular Activities) (Santos 2006) and are involved in a wide range of activities such as proteolysis, protein folding, membrane trafficking, cytoskeleton regulation, organelle biogenesis, transcription control, and microtubule regulation. AAA+ *ATPase* family members are defined by common structural and functional motifs, including Walker A and B motifs and the second region of homology (SRH) (Wendler et al. 2012). The Walker A motif, which is known as the P-loop, binds to phosphates in NTP. The Walker B motif is associated with Mg^{2+}. These 2 motifs are cooperatively involved in NTP hydrolysis (Hanson and Whiteheart 2005). The SRH domain contributes to the maintenance of *ATPase* activity. Mutations to the conserved amino acid residues significantly decrease *ATPase* activity (Karata et al. 1999).The SRH domain is characteristic of the AAA protein family. It does not exist in the Walker-type *ATPases*, which have only 2 consensus motifs, Walker A and B （Karata et al.1999). The AAA-type *ATPases* are widely conserved in archaebacteria, prokaryotes, and eukaryotes, suggesting that they play a critical role in cellular activities. They are involved in various cellular activities and can thus be divided into several subfamilies according to their biochemical activities and physiological functions, including proteolytic activity, proteasome functions, vesicle-mediated secretion, membrane fusion, peroxisome biogenesis, and mitochondrial

functions (Karata et al. 1999; Latterich and Patel 1998; Lupas and Martin 2002). More than 60 AAA-type *ATPase* genes have been identified in the Arabidopsis genome (Sugimoto et al. 2004). They regulate diverse aspects of cellular function, such as protein degradation (Lindahl et al. 1996), 26S proteasome activity (Fu et al. 1999), vesicle trafficking (Rancour et al. 2002), peroxisome biogenesis (Olsen 1998), and hypersensitive responses in plants (Olsen 1998; Sugimoto et al. 2004) , Some have been identified in Helianthus *annus* (sunflower), *Oryza sativa*(rice)(Santos 2006). Historically, the porcine VCP (valosin containing protein) was the first AAA protein whose sequence was determined(Peters et al. 1990). It was found to have high similarity with proteins like p97 from *Xenopus laevis*, which participates in membrane fusion events and CDC48 (cell division control protein) from *Saccharomyces cerevisiae* whose mutants show arrest in mitosis(Koller and Brownstein 1987). All these proteins contain two AAA domains. Moreover, structural studies showed that they form homo-hexametric rings. Later, several other homologs displaying the same characteristics were described, e.g. VAT from T. acidophilum (Thermoplasma acidophilum)(Feiler et al. 1995). AAA proteins with a single AAA domain were also identified and the AAA family was divided into two groups: type I, containing a single copy of the AAA cassette (e.g., Vps4p, FtsH and katanin), and type II, with two AAA cassettes (e.g., p97, Cdc48 and NSF). Involvement of the *ATPase* gene in abiotic stress has been reported previously, such that the gene in *Arabidopsis thaliana* (At1g64110) was inducible by MeJA and wounding, heat, cold and drought, as well as NaCl, ABA and mannitol(Ali et al. 2013).V-*ATPase* is a stress response enzyme present throughout the plant kingdom that serves a vital role in plant protection to adverse environmental conditions, such as salt and drought stress (Gao et al. 2011). Moreover, three isoforms of vacuolar ATPase subunit c (*VHA-c*) from Pennisetum glaucum are regulated in a tissue-specific manner under salinity stress(Tyagi et al. 2006).The previous reports on *At-ECA*1(Endomembrane type Calcium ATPase) demonstrated the role this gene played in providing tolerance to manganese toxicity, Based on their stress-induced expression levels, P-type IICa^{2+}ATPases have also been predicted to act in abiotic and biotic stress adaptation(Kamrul Huda et al. 2013; Wu et al. 2002).For instance, the PCA1 relative mRNA level was up-regulated under dehydration, NaCl and abscisic acid treatments. In a previous study had reported that a P-type ATPase required for rice blast disease and induction of host resistance (Gilbert et al. 2006) and another P-type IIB Ca2+ATPase(OsACA6),which, promotes salinity and drought stress tolerance in tobacco by ROS scavenging and enhancing the expression of stress-responsive genes(Huda et al. 2013). Above all, we supposed to the AAA+ATPase may involve in abiotic stresses in rice. In this report, GeneChip rice genome array and real-time quantitative polymerase chain reaction (PCR) were used to screen a stress tolerance candidate gene *OsATPase* from cultivar Pei'ai64s which is the maternal parent of the super hybrid rice Liang-You-Pei-Jiu (LYP9). Among the genes identified as responsive to stresses, the *OsATPase* gene encoding *ATPase*-induced protein was highly induced by cold stress in the leaves of seeding and panicle of booting stages, which is relevant to stress resistance and was chosen for further study.

Results

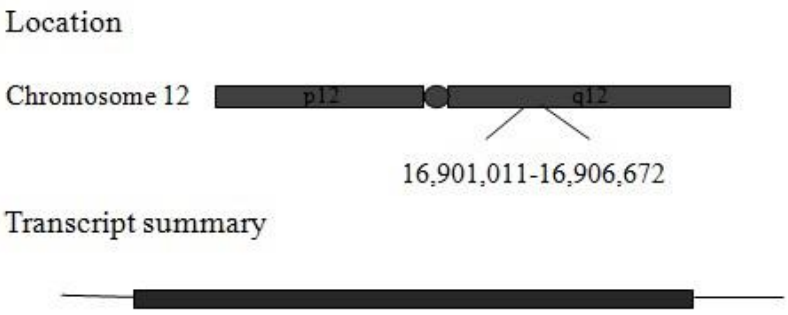
Cloning and sequence analysis of OsATPase

Using Gene Chip Rice Genome Array (Affymetrix) representing 51, 279 transcripts from japonica and indica rice, we indentified *OsATPase*, a gene highly induced by these stresses. To further analyze *OsATPase*, we designed and synthesized two specific primers based on the conserved region after searching GenBank, and then cloned the cDNA sequence of *OsATPase* containing the complete ORF by RT-PCR from rice Pei'ai 64S.*OsATPase*, which is located in chromosome 12, does not contain any introns (Fig.1A). Sequence analysis showed that the cloned cDNA is 1855 base-pair (bp) in length containing a 1587 base-pair (bp) ORF which encodes a protein of 528 amino acids and shared 99.64% identity to the corresponding sequence of *Nipponbare* (Genbank accession: NM_001060322). The 1500 base-pair (bp) upstream promoter sequences from the ATG site of these *OsATPase* genes used the Plant-CARE database and online Plant-CARE software analysis. The possible promoter region (*Nipponbare*: 12:16907057:16912718:1) contains about 30 different *cis*-acting elements; there may be 12 sequences related to stress. Stress inducible related to *cis*-acting elements: 2ARE (*cis*-acting regulatory element essential for the anaerobic induction), 2TC-rich repeats (*cis*-acting element involved in defense and stress responsiveness),3TCA-elements (*cis*-acting element involved in salicylic acid responsiveness), 3Box4 (part of a conserved DNA module involved in light responsive), 1CAT-box (*cis*-acting regulatory element related to meristem expression), 2CCAAT-boxes (MYBHv1 binding site), 35CAATboxes (common *cis*-acting element in promoter and enhancer regions), 1MBS (MYB binding site involved in drought-inducibility), 2GCN4_motifs (*cis*-regulatory element involved in endosperm expression), 1O2-site (*cis*-acting regulatory element involved in zein metabolism regulation), 2Skn-1_motifs (*cis*-acting regulatory element required for endosperm expression), 1 Sp1(light responsive element) and so on(Fig.1B). The existence of theses stress related *cis*-elements, showed that with the promoter region of *OsATPase* responses to various kinds of stress signals, the expression of *OsATPase* is regulated by several stress factors. In order to get protein structure information of *OsATPase*, protein prediction software online was used to deduce its secondary structure (Fig.2), and there were 1 AAA+ *ATPase* domain, 1*ATPase*, AAA-type, core, 1AAA-type*ATPase*, N-terminal domain,1Unintegrated, 2 PANTHER, 1SUPERFAMILY.

Phylogenetic analysis of OsATPase

The database search and analysis with BLASTp through the NCBI website showed that the deduced amino acid sequence of *OsATPase* has higher homology (99%) with *ATPase* (ABA98449.1) from j*aponica* and has 79% homology with OsI_38277(EEC69251.1) from *indica*. BLASTp showed that *OsATPase* has the highest identity with *ATPase* from other plant species. The percentages of identity were from 70 to 50%, such as 70% *OsATPase*2 (ABA98445.1), 54%*HvATPase2*(AAV49983.1),55%*TaATPase* (ACJ22514.1), 55%*TTATPase* (CAH10065.1), 53% *BdBCS*1-like (XP_003578011.1), 52% *BsATPase* (ACO87685.1), 54% *SORBIDRAFT*_08g023140 (XP_002443666.1), 52% *ZmATPase*3 (gb|ACG28844.1), 50% *VvFtsH*-like (XP_002275823.2), respectively (Fig. 3). Comparison of *OsATPase* with several similar proteins from rice and other plant species revealed that AAA+*ATPase* domains were highly conserved in these proteins, suggesting that they have the same functions, probably associated with response to stress. A list of putative *ATPase* proteins was compiled by searching databases for ORFs with characteristic sequences that are highly conserved among AAA+*ATPase* domains. The full-length amino acid sequences of *OsATPase*, several putative rice AAA+*ATPase* gene family members and the

(A)



(B)

-1500 ATTATTTTTAG……...TTCACTTTAAACCATTTAGAAAAATA……
ARE
-1183 ACTATTGTTGTATTCATAGTTAAACCAACAATCTAAAAA……
ARE
-763 AACCGTAGTCTCTAACTTCCGGCATGCAACTTTCAGAAA……
TC-rich repeats
-523 ATCAAGAATATGAGATATGCTAACTGGAAGATTAAAACCAAAAGAGA……
MBS
-303 TGATTCATCAGAACTAGCTATTTTCTTTTAAAAAAGATAGTATAAATAACACAT……
TCA-element
-143 AAGTCAAATCACCTCGCTCGCTTTGCTT……
CAAT-box
-53 GCGCCACAATCGCGCCAATTATATAAGCTGAGCCACACCGCCATAGACCAAGCA……
TATA-box +1

Fig 1. Location of *OsATPase* in rice genome and candidate *cis*-elements of *OsATPase* in the putative promoter region (the putative start codon ATG is denoted with +1). (A) chromosomal location and genomic structure of *OsATPase* gene. (B) Sequence of *OsATPase*, candidate *cis*-elements in the putative promoter region identified through the PlantCARE software. The putative translation initiation site (ATG) is underlined with continuous lines. ARE (anaerobic responsiveness), MBS (MYB Binding Site), TCA-element, CAAT-box, TATA-box, TC-rich repeats and ARE is a *cis*-elements involved in anaerobic induction responsiveness, drought condition, and salicylic acid, respectively. "…" represents bases without print

corresponding genes in other plant species were used to generate a phylogenetic tree. The full-length protein sequences were searched from NCBI, Gramene and Rice Genome Annotation Project (Fig.4). The dendrogram indicates that *OsATPase* has the highest homology with *ATPase* (ABA98449.1) (*Oryza sativa Japonica* Group) and LOC_Os012g28590.1 (rice, putative *ATPase* protein). The sequence divergence of *OsATPase* from other plant species and its relative high similarity to LOC_Os12g28590.1 of rice suggests that *OsATPase* is a putative stress ripening gene.

Expression analysis of OsATPase

In order to identify genes related to cold, drought and heat stresses, the GeneChip rice genome array (Affymetrix) representing 51, 279 transcripts from *japonica* and *indica* rice was used to analyze expression levels of the whole genome of super hybrid rice maternal plant Pei'ai64s in leaves and panicles of seedling, booting and flowering stage, and obtained the up-regulated or down-regulated expression gene. *OsATPase* is one of these genes, in which the expression levels of leaves and spikes are compared with that of the control in the seedling, booting, heading and flowering stage under low-temperature, high temperature and drought conditions according to microarray analysis, at low temperature and drought conditions of up-regulated expression, while in high temperature of down-regulated expression (Fig.5). Low temperature conditions of the booting stage reduced the expression with 0.79-fold in leaves and heading and flowering stage 0.41-fold in panicle, while the up-regulated expression of the seeding 48.83 times in leaves and booting in panicle was 4.47 times. Under drought conditions, the expression level of *OsATPase* up-regulated slightly in the panicle of booting stage, but down-regulate in the leaves and panicles, and seedlings, heading and flowering stage, so *OsATPase* may be insensitive to water-deficiency. While, under high temperature conditions, the average was 0.28 times lower, the maximum was 0.67 times lower, and the minimum was 0.04 times lower. The expression profile of *OsATPase* obtained by the microarray analysis was verified by quantitative real-time RT-PCR (Fig. 5). The qRT-PCR result showed that expression levels of *OsATPase* were

(A)

MASVETWVGFGSALAGVGLLWSRMPEHVHDEARYIISSLVPMAISYFNPYEQITVSEYGE
ERFRRNKMFDAVSTYLSRVCAGGACKLKAELCNNGRDDPIVTLDENQEVVDSFDGARMWW
RLCPKASKNKGAITVTYYPGEADKPRCFKLVFHKRHRQLVLNSYLPSVVRRWRELTAMNR
QRRLFTNHANEAKKSVWTSVPYNPPATFDMLAMDHAKKVEIVDDLTTFQKGKEYHSKVGK
AWKRGYLLHGPPGTGKSTMIGAMANFLDYDVYDLDLTSVKNNSELRKLFLDTTDKSIIVI
EDIDAIEVELTTKRKGKKAANGDEIHDKRMLIEFSDKNDEKSKVTLSGLLSFVDGLWSAC
GSERIFMFTTNHIDRLDPALIRPGRMDKHIEMSYCRFEAFKVLAKSYLDITEHSLFAEIE
RLLDDTDTTPADVANNLMLRSKRNGEISRLLDEIDGAPRADVAKWCKRKRDTDECLAGFV
EILKKSKMESATTPMDSMEEAKEERPNAKESYKMGRIYDENYRLGNAS

(B)

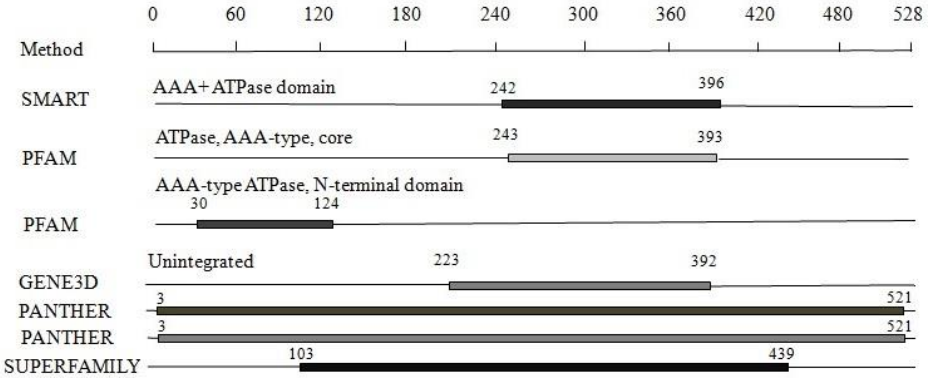


Fig 2. The deduced open reading frame (ORF) and secondary structure of the *OsATPase* protein. (A) The deduced ORF and the AAA Motif (the pane of red) of *OsATPase* protein through the (http://prosite.expasy.org/scanprosite/) web site. (B) The visual map of the structure information of the *OsATPase* protein.

increased at low temperature and high temperature, the expression pattern was generally similar to that revealed by the microarray analysis, suggesting that *OsATPase* is a multiple stress-responsive gene in rice. However, some variations in the amplitude of expression levels were observed between the two sets of data. This most likely resulted from differences in the technologies used and also from the use of the plant materials sampled at different times. Other gene expression data are available in a public microarray database, the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; (Edgar et al. 2002)): the comparisons of tissues (that is, seedlings, ovary, leaf, inflorescence and seed) versus untreated control(NCBI GEO accession no.GSE6893), abiotic stresses (that is, drought, salt and cold) versus untreated control (GSE6901), transzeatin versus mock dimethyl sulfoxide in root and leaves at 30 and 120 min after treatment (GSE6737 Figure 6; Xue et al., 2008).

Discussion

Adenosine triphosphatases (*ATPases*) belonging to the AAA protein family (*ATPases* Associated with various cellular Activities) are involved in a wide range of activities such as proteolysis, protein folding, membrane trafficking, cytoskeleton regulation, organelle biogenesis, transcription control, and microtubule regulation (Santos, 2006). In plants as in mammals, more than hundred genes encoding AAA proteins have been registered to date, e.g. in Arabidopsis thaliana more than 60 cases have been found. The records are restricted to the proteolytic pathway mediated by the 26S proteasome (Rockel et al. 2002), the metalloprotease FtsH (Ogura and Wilkinson 2001), NSF (Lindahl et al. 1996), and PEX6 (Rancour et al. 2002). Some orthologues of the mammalian PEX6 protein have been identified in *Helianthus annus* (sunflower), *Oryza sativa* (rice) (Santos, 2006). Lastly, another group (Sugimoto et al. 2004) has also found the expression of an AAA protein in *Nicotiana tabacumunder* a hypersensitive response (HR). In our study, we analyzed the cDNA sequence of *OsATPase* gene was cloned and its upstream promoter region which contain the *cis*-elements may involve in stress (Fig.1). The result shows that it was cloned successfully, and several *cis*-elements that responded to abiotic stresses were found in the promoter region. Among these *cis*-acting elements, there Are 2AREs (*cis*-acting regulatory element essential for the anaerobic induction), 2TC-rich repeats (*cis*-acting element involved in defense and stress responsiveness), 1MBS (MYB binding site involved in drought-inducibility), 3TCA elements (Chen et al. 2005; Lim et al. 2006) (*cis*-acting element involved in salicylic acid responsiveness) and so on , which shows that the gene may responses to multiple stresses and tolerance. The protein structure determines the function, from the NCBI, GRAMENE and trans- membrane helix prediction (TMHMM); the *OsATPase* protein contains one AAA-type *ATPase* N-terminal domain, one AAA+*ATPase* domain and one of the three *ATPase* relative domains is AAA-superfamily, which indicated that *OsATPase* gene encodes a protein similar to *OsATPase* and it belongs to AAA family (Fig.2). *ATPases* that bind and hydrolyze ATP and other nucleotides (GTP, CTP, and, UTP), play an important role in converting chemically stored energy into a biological activity (Santos, 2006).Through the NCBI gene prediction on *OsATPase* protein active sites, superfamily regional and other species of the conserved region of the forecast comparison showed that *OsATPase* gene protein is one of AAA (*ATPases* Associated with various cellular

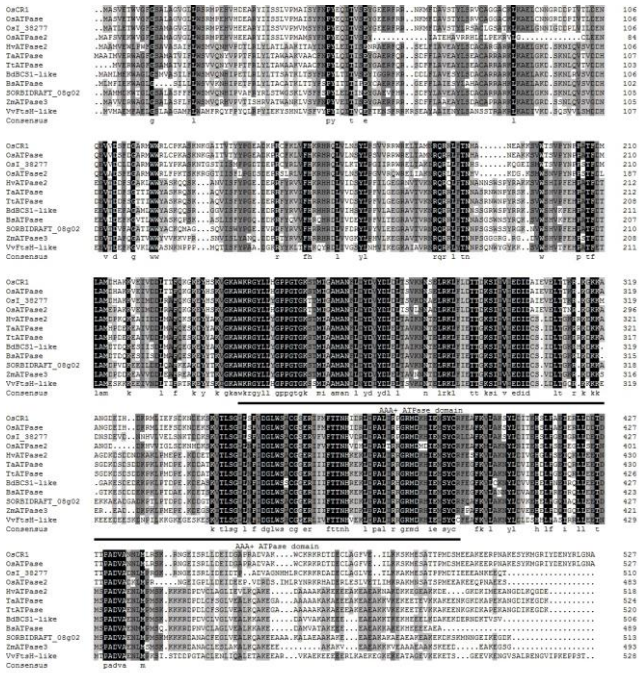


Fig 3. Multipe amino acid sequence alignment of the *ATPase* proteins and corresponding protein sequences based on OsCR1 domain sequences using the Clustalw program. The underline indicted ATPase domain. *OsCR*1(lcl|60461) [*Oryza sativa Japonica* Group]; *OsATPase*(ABA98449.1) [*Oryza sativa Japonica* Group]; *OsI*_38277(EEC69251.1)[*Oryza sativa indica* Group]; *OsATPase*2(ABA98445.1)[*Oryza sativa Japonica* Group]; *HvATPase*2 (AAV49983.1) [*Hordeum vulgare subsp. vulgare*]; *TaATPase* (ACJ22514.1)[*Triticum aestivum*]; *TTATPase* (CAH10065.1) [*Triticum turgidum*]; *BdBCS1*-like (XP_003578011.1) [*Brachypodium distachyon*]; *BsATPase* (ACO87685.1) [*Brachypodium sylvaticum*]; *SORBIDRAFT*_08g023140 (XP_002443666.1)[*Sorghum bicolor*];*ZmATPase*3 (gb|ACG28844.1)[Zea mays]; *VvFtsH*-like (XP_002275823.2)[*Vitis vinifera*].

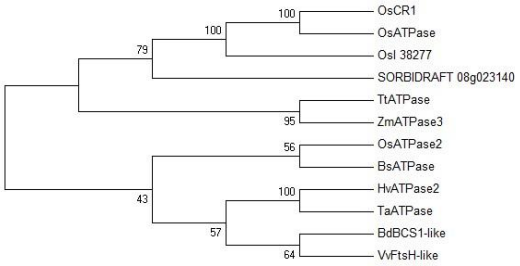


Fig 4. Phylogenic-tree of the *OsATPase* amino acid sequence with other plant *ATPase* proteins and corresponding sequences from rice. The phylogenetic test used was the Bootstrap test by Maximum Parsimony method, as implemented by MEGA version 5.02. Dendogram branches are labeled with percentage of 1,000 iterations supporting each branch. Bootstrap values are shown in the nodes of the tree.

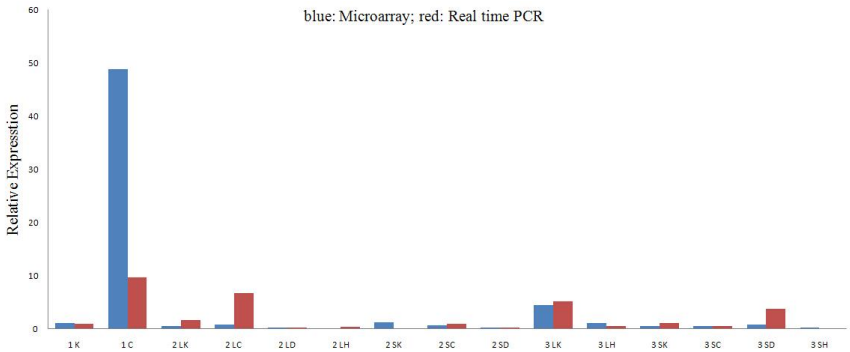


Fig 5. Relative expression of *OsATPase* in leaves and panicles of *indica* rice cultivar Pei'ai 64S under the various stresses and in normal growth conditions, at different developmental stages.1: Seedling stage; 2: booting stage; 3: heading and flowering stage; L: leaf; P: panicle; K: control; C: cold; H: heat; D: drought.

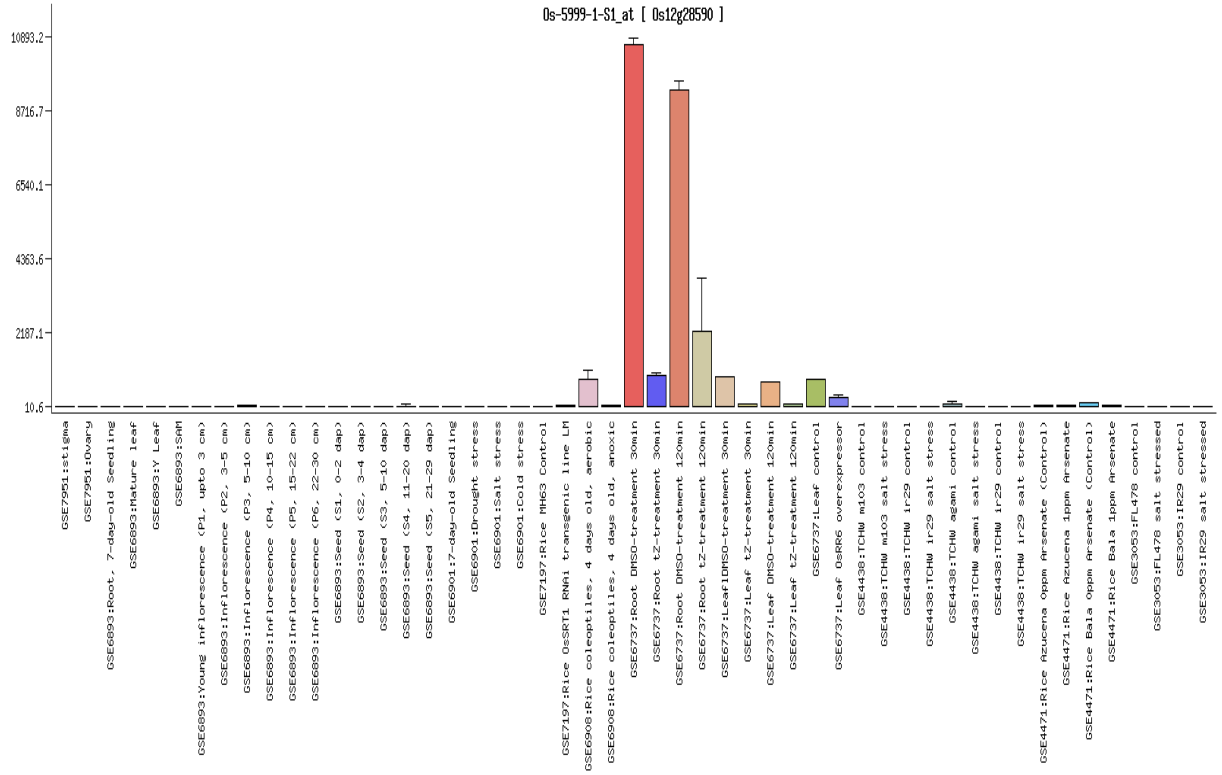


Fig 6. RiceGE: Gene expression atlas for *OsATPase*. X axis: different series and relative tissues; Y axis: total RNA expression level. GSE7951 genome-wide gene expression profiling of rice stigma [*O. sativa*]; GSE3053 rice salt expression [*O. sativa* (*indica* cultivar-group)]; GSE4438 Expression data from rice under salinity stress [*O. sativa* (*indica cultivar*-group) O. sativa (*japonica cultivar* -group)]; GSE4471Expression data from rice varieties Azucena and Bala grown in 0 and 1 ppm arsenate [*O. sativa*]; GSE6737 Over-expression of a Type-A response regulator alters rice morphology and cytokinin metabolism [*O. sativa*]; GSE6893 expression data for reproductive development in rice [*O. sativa*]; GSE6901 expression data for stress treatment in rice seedlings [*O. sativa*];GSE6908 transcript profiling of the aerobic and anoxic rice coleoptiles [*O. sativ*a]; GSE7197 down-regulation of OsSRT1 induces DNA fragmentation and cell death in rice [*O. sativa*]. GEO: Gene expression omnibus; SAM:shoot apical meristem; tZ: trans-zeatin; DMSO: dimethyl sulphoxide; web link: platform gpl2025.

Activities) (Santos, 2006). Predicted sequence length and other species have high similarity. Analysis of the phylogenetic trees displays the evolutionary relationship among 114 *OsATPase* genes of *O.sativa indica cultivar*-group and divergence in plants. The GeneChip rice genome array and quantitative real-time RT-PCR analysis showed that *OsATPase* was highly expressed in the seedling leaves, panicles at booting stage after a short cold treatment, and also slightly induced by drought conditions in booting and heading stage panicle (Fig.5).The maximum up-regulation in the leaf under cold treatment with GeneChip rice genome array and real-time quantitative PCR was 48.83- and 9.67-fold, while in panicle under drought treatment, the maximum up-regulation was 3.70- and 1.02-fold, respectively. Researches find that *ATPase* genes seem to be involved in processes of plant development and signaling pathway. Previously, some reported about *ATPase* involved in responses to abiotic stresses, such as the transgenic yeast expressing vacular ATPase increased salt, drought, ultraviolet (UV), oxidative, heavy metal, cold and high temperature tolerance (Gao et al. 2011). V-*ATPase* is a stress response enzyme present throughout the plant kingdom that serves a vital role in plant protection to adverse environmental conditions, such as salt and drought stress(Gao et al. 2011). The Plant Ca^{2+}*ATPases* are members of the P-type *ATPase* superfamily and play essential roles in pollen tube growth, vegetative development, inflorescence architecture, stomata opening or closing as well as transport of Ca^{2+},$Mn^{2+}$and Zn^{2+}. Their role in abiotic stress adaptation by activation of different signaling pathways is emerging (Kamrul Huda et al. 2013). Also, in *Arabidopsis*, *ATPase* gene was inducible by MeJA and wounding, heat, cold and drought, as well as NaCl, ABA and mannitol, which, suggesting that *ATPase* gene involved in abiotic stress responses. Because of the limitations, it was not possible to treat materials in each tissue of the developmental stages to analyze its expression level, but choose leaf and panicle in one of the development stage under one to three stress treatments to analyze expression patterns of *OsATPase*, did not affect global recognition of its response to stress treatments. And further analysis to stresses can use real-time quantitative PCR to implement. We also searched the web (http://signal.salk.edu/) and found RiceGE: Gene Expression Atlas of *OsATPase* (Os-5999-1-S1_at). The results show that the expression level of *OsATPase* was up-regulated obviously under DMSO treatment with the rice root, which was in variations with the results of GeneChip rice genome array (Affymetrix) and real-time quantitative PCR (Fig.7). This work describes the identification and molecular characterization of a new low-temperature regulated gene *OsATPase* from Pei'ai64s, whose expression in leaves of seedling, panicle of booting stage was up-regulated obviously after cold treatment(Santos 2006; Saraste et al. 1990). To further analyze the function of protein, we need to continue a research into the expression of the gene under other stress and the phenotype in transgenic plants.

Materials and Methods

Plant materials growth, management

The germinal seeds of cultivated rice Pei'ai 64S (O. sativa L.) were suspended in a sterile solution of 0.1% $HgCl_2$ for 10 min, washed 3 times using running water, immersed for 3 days under 25°C and changed water once a day, then were germinated and grown in distilled water at 37°C for 2 to 3 days. They were broadcasted in batches in net basin of Institute of Subtropical Agriculture, Chinese Academy of Sciences. Plants were divided into one control and two treatment groups. The control group was maintained under normal growth conditions and the treatment groups were exposed to drought, heat and cold stresses. At five-leaf stage, part of them are took as the test material of the seedling stage; other parts, as test material of booting and flowering stage, are transplanted to the other pots, 5 plants each pot, and was put in the greenhouse under natural conditions, regular water and fertilizer management, pest and disease control.

Rice cold, heat and water-deficit treatment

For the drought tests the watering was avoided to the basin until the treatment group dried out. Meanwhile, the control group was also put in the dry shed, but with water in pots, then the leaves were harvested when they started curling after 16 h. For the heat tests, materials were put in climate incubator, PGC15.5 (Percival, USA) for 2 h under 45°C, while the control group was put into PGC15.5 under 45°C. For cold test, we placed the seedlings into PGC15.5 for 12h under 4°C at booting and heading stage for 16h under 12°C. The control group was placed into another PGC15.5, and both control and treatment group was under dark conditions.

Sample preparation for RNA

Four or five countdown second leaves were collected from treatment and control group and four or five leaves were harvested which were not out of the young panicle or the middle of spiked out flower. The materials were cut into pieces, then ground into a dry powder with liquid nitrogen and immediately divided into preinstalled 1.0 ml Trizol extraction (Invitrogen) in 1.5 ml centrifugal tubes, about 100 mg each tube. We used low temperature marker pen to mark labels, tightly closed lid, then vigorously vortex to sure that the samples were mixed with TRIzol extraction, seal the tubes with Parafilm wrap. The samples were stored at -70°C until required.

Isolation of total RNA and cDNA cloning

Total RNA was isolated from the frozen samples using TRIzol. Extract method using TRIzol reagent (Invitrogen). The samples saved in -70°C were taken out; vortexed to homogeneity, chloroform (200µl) was added, vigorously shaken for 15s and then centrifuged at12 000×g for 15 min at 4°C. The upper layer was carefully removed from each tube, was transferred to another centrifuge tube, isopropanol(500µl) was added, precipitated for at least 1 h at -40°C, then centrifuged to separate the RNA. The RNA pellets were twice washed by 75% ethanol, air dried and dissolved in the appropriate volume of RNase-free water. The purity of RNA was determined by theA260/280 absorbance ratio (1.9 to 2.0). Isolated RNAs were stored at -70°C, after checking the purity and integrity of 18S, 5S and 28S rRNA bands on 1.5% agarose gel. Full-length of *OsATPase* cDNA was amplified using high fidelity HiFi taq DNA polymerase (TransGen). Special primers were designed using the software primer-premier 5.0 after searching homology cDNA sequence that was *OsATPase*-F: 5'- TCCACACAACGGCAATTA-ATC-3' with a unique Hind III restriction site upstream from the translational start codon, *OsATPase*-R: 5'-TCTAGCTGGCATTTCCCAAC -3' with a unique BamH I restriction site downstream from the termination codon. The PCR cycler was programmed as follows: an initial denaturation for 5 min at 94°C, 30 amplification cycles, [30s at 94°C (denaturation), 30s at 60°C(annealing), and 1.5min at 72°C (polymerization)], followed by a final elongation for 5 min at 72°C. All the PCR products were purified using Gel Extraction Mini Kit (Biomed, China), the amplified product was ligated into vector pMD18-T (TaKaRa, Dalian, China), then cloned into Escherichia coli strain Top10. The positive transformants were screened by using ampicillin selection. And restriction enzymes Hind III and BamH I were used for double cuts for confirmation. Restricted fragments were analyzed on 1.0% agarose gel. Positively screened clone was sequenced by invitrogen.

Microarray data analysis and real-time quantitative PCR analysis

The process was according to protocols previously described by Xu, Jiang, Dong and Chen(Chen et al. 2012; Dong et al. 2011; Jiang et al. 2011; XU 2008).Briefly, according to Affymetrix expression microarray experiments manual provided by GeneTech Biotechnology Limited Company, Shanghai, the following steps are: (1)Total RNA extraction and purification; (2)cDNA synthesis and purification;(3) cRNA synthesis and transcription purification in vivo;(4) cRNA fragmentation, preparation of hybridization solution;(5) chip hybridization;(6) elution chip;(7) scan chips; (8) data analysis. Expression levels of *OsATPase* in the microarray were verified further by real-time qRT-PCR. Total RNA was extracted with Trizol reagent. Prior to qRT-PCR, RNA was diluted to 20ng/ul. All primers were designed through software Primer Expression 3.0. For quantitative real-time PCR, SYBR Green RT-PCR One Step Kit (Qiagen, Cat. No. 204243) and fluorescent real-time quantitative PCR instrument was used to detect the PCR products, and used 18S as β-actin. According to the sequence information obtained from the Gramene (http://www.gramene.org/), we designed the primer pairs used in the real-time PCR. *OsATPase*-F: 5′-TGTTCACGACGAACCACATTG–3′,OsATPase-R:5′-AGCGGCAGTAAGACATTTCGA-3′; 18S-F:5′-CGTCCCTG CCCTTTGTACAC-3′, 18S-R:5′-CGAACACTTCACCGGA TCATT-3′.The relative expression levels of *OsATPase* were normalized to the 18S gene using the method described by Ma and Chen (Chen et al. 2011; Ma et al. 2009).

Sequence analysis

The analysis and comparison of the deduced amino acid sequence with published sequences were performed with BLASTp (Standard Protein-Protein BLAST) on the NCBI server (http://www.ncbi. nlm.nih.gov/). Promoter analysis of 1500bp, upstream of OsATPase gene, was performed with Plant CARE on the web (http://bioinformatics.psb.ugent.be/webtools/plantc-are/html/). Conserved domains in OsATPase were identified with online protein predict software InterProScan (http://www.ebi.ac.uk/InterProScan/). Protein prediction software online (http://www.gramene.org/Oryza_sativa/

Transcript/Protein Summary? db=core; g=OS12G0471100; h=BLAST_NEW:BLA_2nfcH0SC6!!20130510; r=12:16907057-16912718; t=OS12T0471100-01) was used to deduce its secondary structure. OsATPase gene was aligned with other AAA proteins from different species using Clustalw program, and then the phylogenetic tree was generated by the Maximum Parsimony method using Mega4.1. Bootstrap (1000 replications).

Statistical analysis

For water deficit experiment, cold and heat all treatment has three replicants and each replicant has at least 5 plants. All these plants were arranged as Random Completed Block Design and rotated twice a week to ensure consistency.MEGA software was performed on the phylogenetic analysis by Maximum Parsimony method, and Clustalw program approach was used for multiple comparisons to check the significant differences.

Conclusions

In this report, we screen a stress tolerance candidate gene *OsATPase* from cultivar Pei'ai64s which is the maternal parent of the super hybrid rice Liang-You-Pei-Jiu (LYP9). We also analyzed the upstream promoter sequence of this gene (*OsATPase*) and found some *cis*-elements involved in abiotic stress. Moreover, we processed multiple sequence alignment among the AAA family and pholygenetic tree analysis. Among the genes identified as responsive to stresses, the OsATPase gene encoding *ATPase*-induced protein was highly induced by cold stress in the leaves of seedling and panicle of booting stages, which is relevant to stress resistance and was chosen for further study.

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