African Journal of Biotechnology Vol. 11(74), pp. 13873-13881, 13 September, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1218
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Isolation of a novel abscisic acid stress ripening (*OsASR*) gene from rice and analysis of the response of this gene to abiotic stresses

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Accepted 17 August, 2012

Abiotic stresses constitute a serious threat to agricultural production, which often develops into major crop production reducing factors around the world. Molecular biology technology has, however, emerged as a promising vehicle improving crop tolerance. A cold-, drought- and heat-inducible gene designated *Oryza sativa* L. abscisic acid stress-ripening (*OsASR)* gene, GenBank accession: AK318549.1 was identified in rice Pei'ai64s (*O. sativa* L. ssp. *Indica* cv.) using the GeneChip rice genome array (Affymetrix) representing 51, 279 transcripts from two rice subspecies *japonica* and *indica*. The expression profile of *OsASR* obtained by the microarray analysis was confirmed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of the gene. The two sets of data matched very well, suggesting that *OsASR* is a multiple stresses responsive gene in rice. Based on the sequence, PCR primers were designed. The cDNA with the whole open reading frame (ORF) was amplified by PCR and cloned. Sequence analysis showed that the cDNA encodes a protein of 284 amino acid residues with M.W. ≈ 11.7 kD and pI ≈ 10.4. The gene encodes a protein with several conserved domains. Comparison of protein sequences indicates that *OsASR* encodes a putative abscisic acid stress-ripening protein. Analysis of the putative promoter region for candidate cis-regulatory elements using PlantCARE software identified seven kinds of cis-elements related to stress responses. Based on the aforementioned analyses and results obtained, we propose that *OsASR* is a novel candidate gene involved in stress tolerance in rice.

Key words: Rice, microarray, abiotic stress, reverse transcription polymerase chain reaction (RT-PCR), abscisic acid stress ripening.

INTRODUCTION

Environment factors, such as high temperature, low temperature, drought and high salinity, greatly limit plant growth and yield potential. These stresses may occur at any stage of plant development and often several types of stresses simultaneously affect plants (Rizhsky et al., 2004). Plants have developed the ability to exhibit a large spectrum of responses to abiotic stresses in order to survive the stress conditions, including a large number of physiological, biochemical and molecular changes (Kalifa et al., 2004b). The molecular and cellular processes underlying the acclimation of higher plants to abiotic stresses have attracted much interest, as environmental stress conditions result in the seriously loss of crop production in many parts of the world (Cushman and Bohnert, 2000; Mittler, 2006). A large number of genes induced by abiotic stresses have been identified recently using molecular and genetic approaches, for example, 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 to regulate gene

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Abbreviations: ORF, Open reading frame; **ASR,** abscisic acid stress and ripening

expression through signal transduction pathways (Xiong et al., 2002; Shinozaki et al., 2003). Research indicates that partial of the plants genome participate in the self-response of drought, cold and heat stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001; Xiong et al., 2002; Shinozaki et al., 2003) and change in the expression of a single gene can enhance the ability of drought, cold and salt stress. Plants respond to environmental challenges in part by altering their gene expression profiles, which ultimately leads to various adaptive responses at the cell and whole-plant levels (Xiong et al., 2001).

Abscisic acid stress and ripening (ASR)-induced proteins are hydrophilic charged low-molecular weight plant-specific proteins, which are regulated by abiotic stresses, abscisic acid (ABA) or during fruit ripening (Dai et al., 2011). Since the first ASR transcripts were discovered in tomato (Iusem et al., 1993), more than 30 ASR-like genes have been identified in different plant species (Fischer et al., 2011). All available data suggest that ASR proteins are encoded by small multigene families: five genes in tomato (Rossi et al., 1996), four genes in loblolly pine (Chang et al., 1996) and six in maize and rice (Frankel et al., 2006). They are from gymnosperm to angiosperm and from monocot to dicot, but surprisingly these genes are not present in *Arabidopsis* (Carrari et al., 2004). The expression patterns of the ASR gene family members vary among different species during plant development and abiotic stresses (Maskin et al., 2001). Tomato ASR1 protein is localized in both the nucleus and the cytosol (Kalifa et al., 2004a). Desiccation and zinc binding can induce increased order in the primary structure and homodimerization in tomato ASR1 protein (Goldgur et al., 2007). The ASR1 protein is unstructured in cytoplasm and may function as chaperone-like protein protector due to their high hydrophilicity (Goldgur et al., 2007; Konrad and Bar-Zvi, 2008). The hydrophilic MpASR protein could protect L-lactate dehydrogenase (L-LDH) from cold-induced aggregation (Dai et al., 2011).

Expression levels of a number of ASR genes are rapidly increased in response to water deficit, cold, salt, limited light (Maskin et al., 2001; Kalifa et al., 2004b), the cloning of maize ASR as genes linked to drought resistance (de Vienne et al., 1999), and the divergence of tomato ASR paralogs due to the adaptation to a hostile environment (Frankel et al., 2003) strongly suggest the role of these proteins in the plant response to environmental cues (Saumonneau et al., 2008). Constitutive expression of MpAsr in *Arabidopsis* helped the plant to reduce cell membrane damage and increase cytoplasmic osmoprotectants in response to stress (Dai et al., 2011). In rice, ASR5 mRNA is up-regulated by cold, drought, high-salinity and ABA (Rabbani et al., 2003). Low-temperature stress increased mRNA levels of OsAsr1 in both vegetative and reproductive organs (Kim et al., 2009).

In this paper, GeneChip rice genome array and real-time quantitative polymerase chain reaction (PCR) were used to screen a stress tolerance candidate gene *OsASR* from cultivar Pei'ai64s which is the maternal parent of the super hybridization rice *Liang-You-Pei-Jiu* (*LYP9*). The *OsASR* gene encoding ASR-induced protein was highly induced by cold stress and heat stress in the leaves of booting and heading stages, and this is relevant to stress resistance.

MATERIAL AND METHODS

Stress treatments, sample preparation, isolation of total RNA, GeneChip rice genome array (Affymetrix) and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

The process was according to protocols previously described by Xu et al. (2008), Jiang et al. (2011, 2012) and Dong et al. (2011). The germinal seeds of cultivated rice Pei'ai 64S (*Oryza sativa* L.) were suspended in a sterile solution of 0.1% $HgCl_2$ for 10 min, washed three times using running water, immersed for three days under 25°C and changed water once a day, then were germinated and grown in distilled water at 37°C for two to three days. Plants were divided into one control and three treatment groups. For the drought tests, the water was poured away from the basin; the treatment groups were put in scaffold to dry out meanwhile the control group was kept at the water level. The leaves were harvested when they started curling after 16 h. For the heat tests, the treatment group was exposed to 45°C for 2 h, then plants were harvested for the cold tests the treatment group was harvested after exposed to 4°C for 12 h at seedling stage and to 12°C for treatment 16 h at booting and flowering stages. All the rice seedlings were kept in a U.S. Percival produced PGC15.5 artificial climate chamber, while the control group was in another chamber at 28°C. Test groups and control groups were in the dark.

Real-time PCR primers were designed by Primer Expression 3.0 software, the target gene (*OsASR*) primers were *OsASRrt*-F:5′-CCA CGA GCA CCA CGA GAA G-3′, *OsASRrt*-R:5′-AGG TAG CAA TGC ACG AAT GGA-3′, amplified fragment length 93 bp. The specific primers for the 18S rDNA gene internal control used were 18S-F: 5′-CGT CCC TGC CCT TTG TAC AC-3′, 18S-R: 5′-CGA ACA CTT CAC CGG ATC ATT-3′.

cDNA cloning

Special primers were designed using the software primer-premier 5.0 after searching homology cDNA sequence, that were *OsASR*-F: 5'-AAG CTT TAG CTA ACT AAA TTT CCC CGT A-3' with a unique Hind III restriction site upstream from the translational start codon *OsASR*-R: 5'-GGA TCC TGG ATG GGA TGG AT T AGT GG-3' with a unique BamH I restriction site downstream from the termination codon. The full-length of *OsASR* cDNA was amplified using high fidelity HiFi taq DNA polymerase (Transgen). The PCR cycler was programmed as follows: an initial denaturation for 5 min at 94°C, 30 amplification cycles, [30 s at 94°C (denaturation), 30 s at 62°C (annealing), and 30 s at 72°C (polymerization)], followed by a final elongation for 10 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 Top 10. The positive transformants were screened by using ampicillin selection, and restriction enzymes Hind III and BamH I were used for double cuts to confirmation. Restricted fragments were analyzed on 1.0%

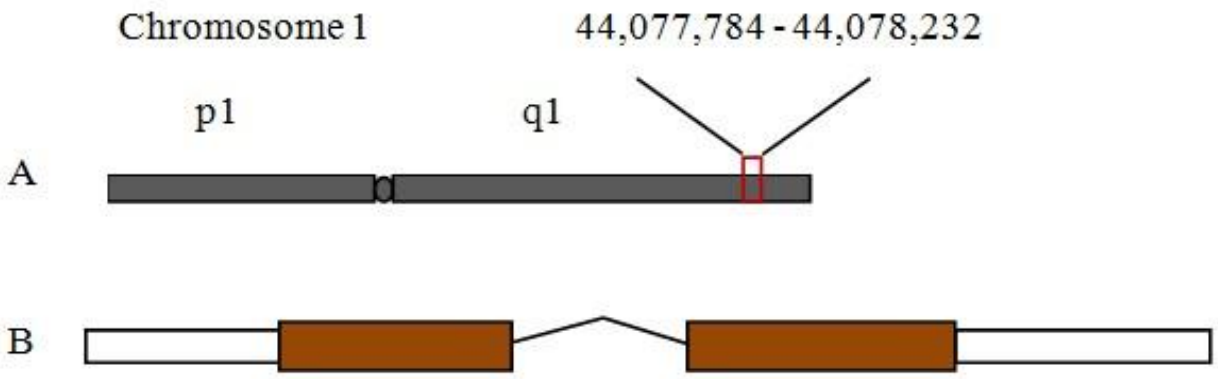

Figure 1. Location and transcript summary of OsASR. (A) Location of OsASR; (B) Transcript summary of OsASR.

agarose gel. Positively screened clone was sequenced by Invitrogen.

Sequence analysis

The genomic sequence and chromosome location of *OsASR* were determined by comparison of the cloned cDNA with the genomic DNA sequences in GenBank. Promoter analysis of 1500 bp, upstream of *OsASR* gene was performed with PlantCARE on the web (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The analysis and comparison of the deduced amino acid sequence with published sequences were performed with basic local alignment search tool proteins (BLASTp, Standard Protein-Protein BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/). Conserved domains in *OsASR* were identified by online protein predict software InterProScan (http://www.ebi.ac.uk/InterProScan/). *OsASR* gene was aligned with other ASR proteins from different species using DNAMAN. Phylogenetic relationship with other ASR proteins from different species was constructed using Mega4.1.

RESULTS

Cloning and sequence analysis of *OsASR*

Based on the conserved region of the *ASR* gene sequence of rice in Gramene and GenBank, two specific primers *(OsASR*-F and *OsASR*-R) were designed and synthesized for the amplification of *OsASR*. The full-length cDNA sequence of *OsASR* was cloned through RT-PCR from rice Pei'ai 64S. Sequence analysis showed that the cloned cDNA is 379 bp in length containing a 315 bp open reading frame (ORF) encodes a protein of 105 amino acids with M.W. ≈ 11.7kD and pI ≈ 10.4. It was consensus with the cDNA (Genbank accession: AK318549.1) of J075129P18 from *Nipponbare*. Comparison of *OsASR* with the mRNA (NM_001185822.1) of Os01g0959200 from *Nipponbare* which publicated in GenBank, there was 1 bp surplus, the similarity was 99.7%. Comparison of the cDNA and its corresponding genomic DNA sequence (NC_008394.4, ranging from positions 44,077,784 to 44,078,232) from GenBank showed that *OsASR* contains one intron and is located at chromosome 1 of rice (Figure 1). Further results show that the cDNA of *OsASR* shared high similarity to several sequences corresponding to genes of unknown functions, including XM_002455560.1 (86%) from Sorghum bicolor and CT829892.1 (84%) from rice (*indica*) Guang Lu Ai 4.

To better understand the organization of the regulatory region of *OsASR* gene, several putative cis-elements related to stress responses were identified in the putative promoter region of *OsASR* about 1.5 kb upstream of the transcriptional start site using PlantCARE (Figure 2). The presence of putative cis-acting elements indicated that the *OsASR* gene might be regulated by the interaction between the cis-acting elements in the promoter and the corresponding trans-acting factors. There were 29 TATA box, 19 CAAT box (common cis-acting element in promoter and enhancer regions), 6 ABA-responsive element (ABRE), 2 Box 4 (part of a conserved DNA module involved in light responsiveness), 1 CCAAT-box (MYBHv1 binding site), one gibberellin-responsive element (GARE-motif), two MYB binding site (MBS, involved in drought-inducibility), one cis-acting regulatory element (RY-element, involved in seed-specific regulation), two auxin-responsive element (TGA-element). The existence of these stress related to cis-elements, provided an evidence that the promoter region of *OsASR* responses to various kinds of stress signals, the expression of *OsASR* is regulated by several stress factors.

To get protein structure information of *OsASR*, protein predict software online (http://www.ebi.ac.uk/InterProScan/) was used to deduce its secondary structure (Figure 3), and find there was one ABA-WDS domain, which is consistent with the sequence alignment result.

Sequence and structural analyses of *OsASR* protein

The database search and analysis with BLASTp showed

Figure 2. Candidate cis-elements in the putative promoter region.

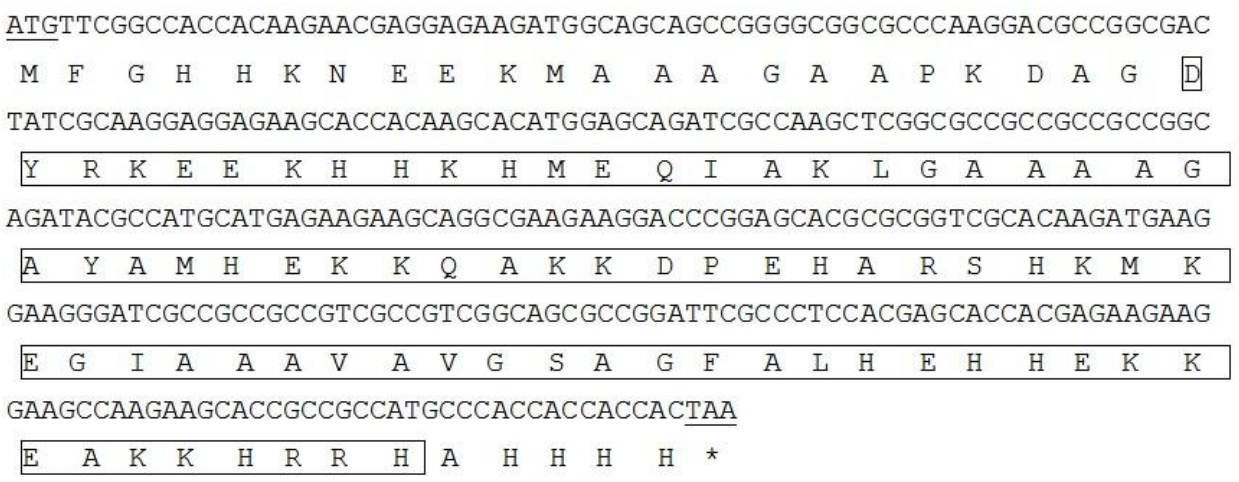

Figure 3. Sequences of *OsASR*, the deduced ORF and secondary structure of *OsASR*.

that the deduced amino acid sequence of *OsASR* was consensus with hypothetical protein OsI_05284 (EEC72202.1) from 93-11 and hypothetical protein OsJ_04835 (EEE56041.1) from *Nipponbare*, and shares highest identity (79.1%) with putative abscisic acid response protein (BAD87118.1) from *Nipponbare*. BLASTp analysis showed that *OsASR* also shares the highest identity with other plant species. The percentages of identity were from 74 to 56%, such as 74% (predicted protein from Hordeum vulgare subsp. vulgare), 69% (SORBIDRAFT_06g016530), 63% (SbASR) and so on (Figure 4). Comparison of *OsASR* (EEC72202.1) with

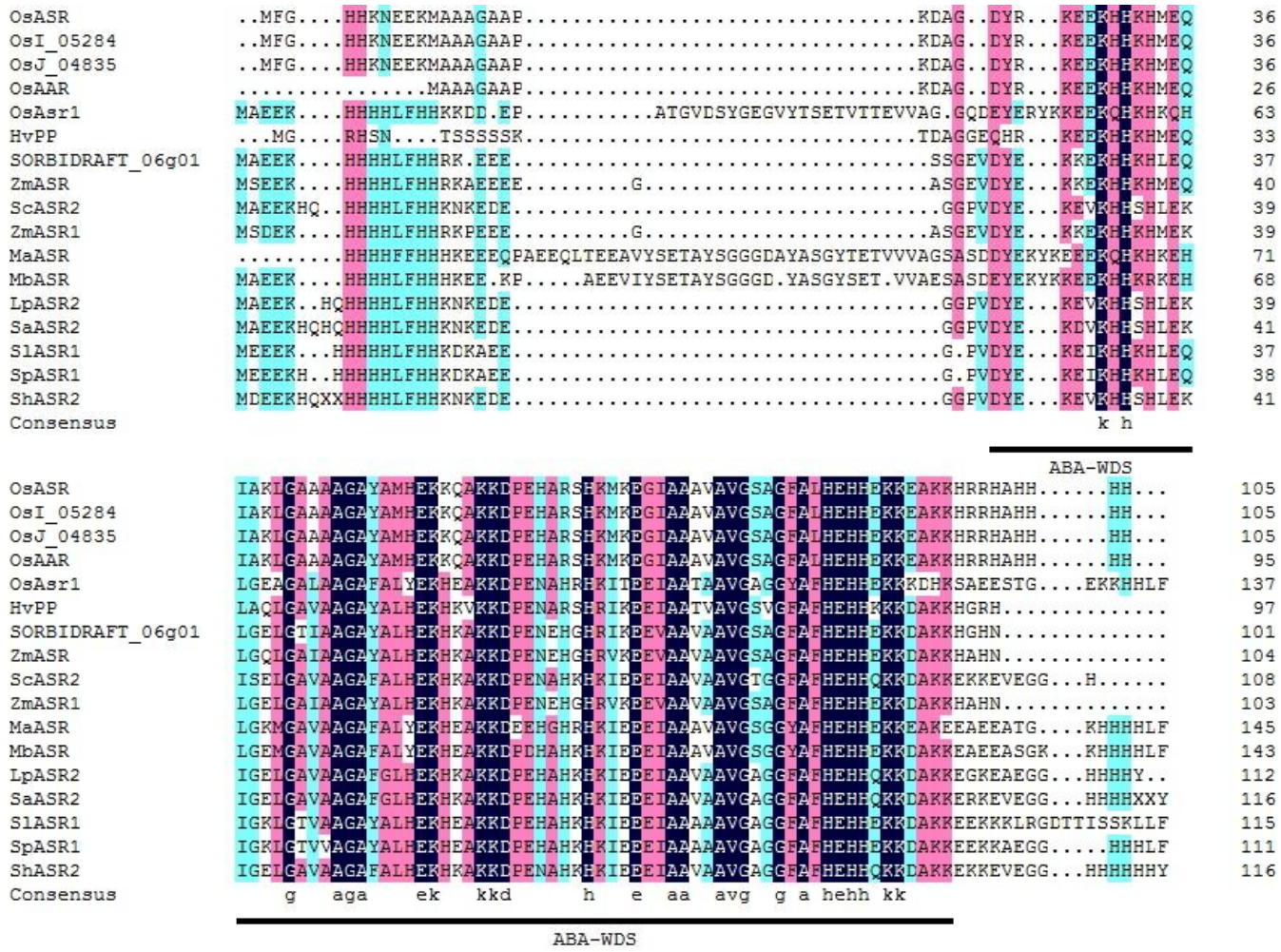

Figure 4. Multiple amino acid sequence alignment of the ASR proteins and corresponding protein sequences.

several similar proteins from rice and other plant species revealed that ABA-WDS domains were highly conserved in these proteins, suggesting that they have the same functions, probably associated with response to stress. A list of putative ASR proteins was compiled by searching databases for ORFs with characteristic sequences that are highly conserved among ABA-WDS domains.

The full-length amino acid sequences of *OsASR*, several putative rice ASR gene family members and the 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 (Figure 5). The dendrogram indicates that *OsASR* has the highest homology with OsJ_04835 (*Nipponbare*, EEE56041.1) and LOC_Os01g72910.1 (rice, putative abscisic stress-ripening protein). The sequence divergence of *OsASR* from other plant species and its relative high similarity to LOC_Os01g72910.1 of rice suggests that *OsASR* is a putative abscisic acid stress ripening gene.

Expression analysis of *OsASR*

In order to reveal 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 upregulate gene *OsASR* (Figure 6). Microarray analysis showed that many genes were up- and down-regulated by multiple stresses in the rice genome. *OsASR* was one of the genes highly induced by cold and heat stresses. The result shows that the expression levels of *OsASR* were elevated in different tissues of rice at different stages under the stresses, with an average increase of 9.17-fold, ranging from 0.11- to 31.72-fold increases. Under cold treatment, the expression level of *OsASR* increased 8.85- and 21.38-fold in the leaf of seedling and booting stages, respectively, and a 8.30-fold increase in the panicle of

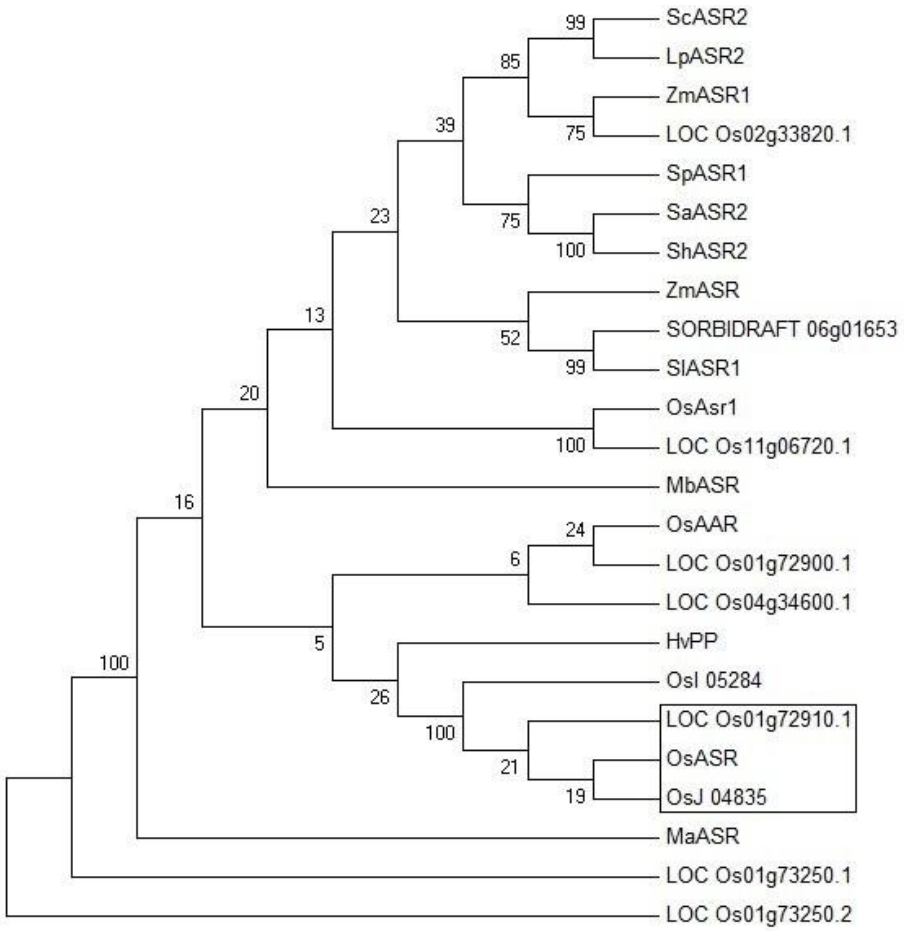

Figure 5. Phylogenic-alignment of the *OsASR* amino acid sequence with other plant ASR proteins and corresponding sequences from rice.

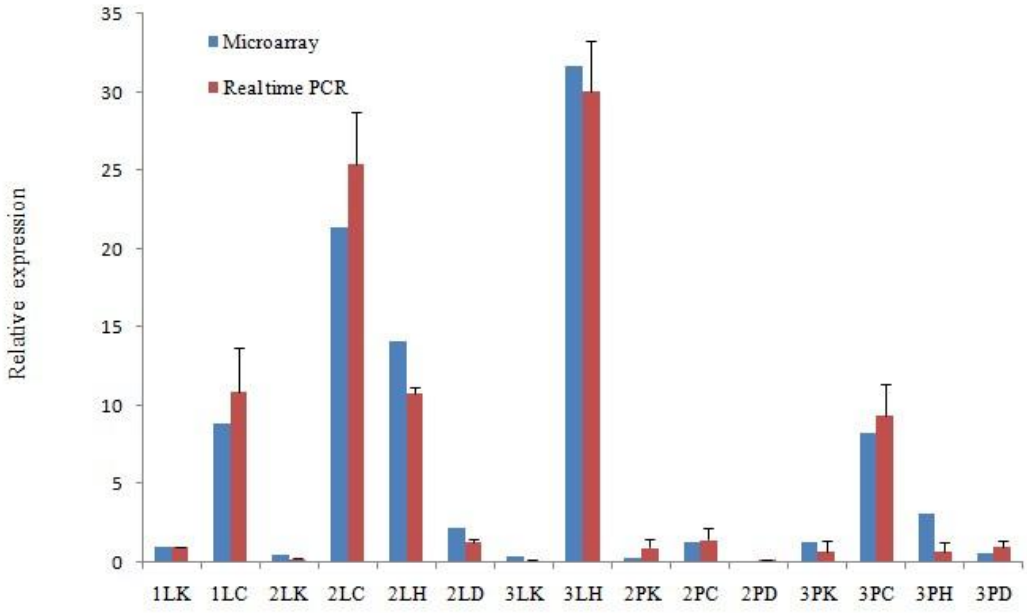

Figure 6. Relative expression levels of *OsASR* in different tissues of Pei'ai64s at different development stages under stresses.

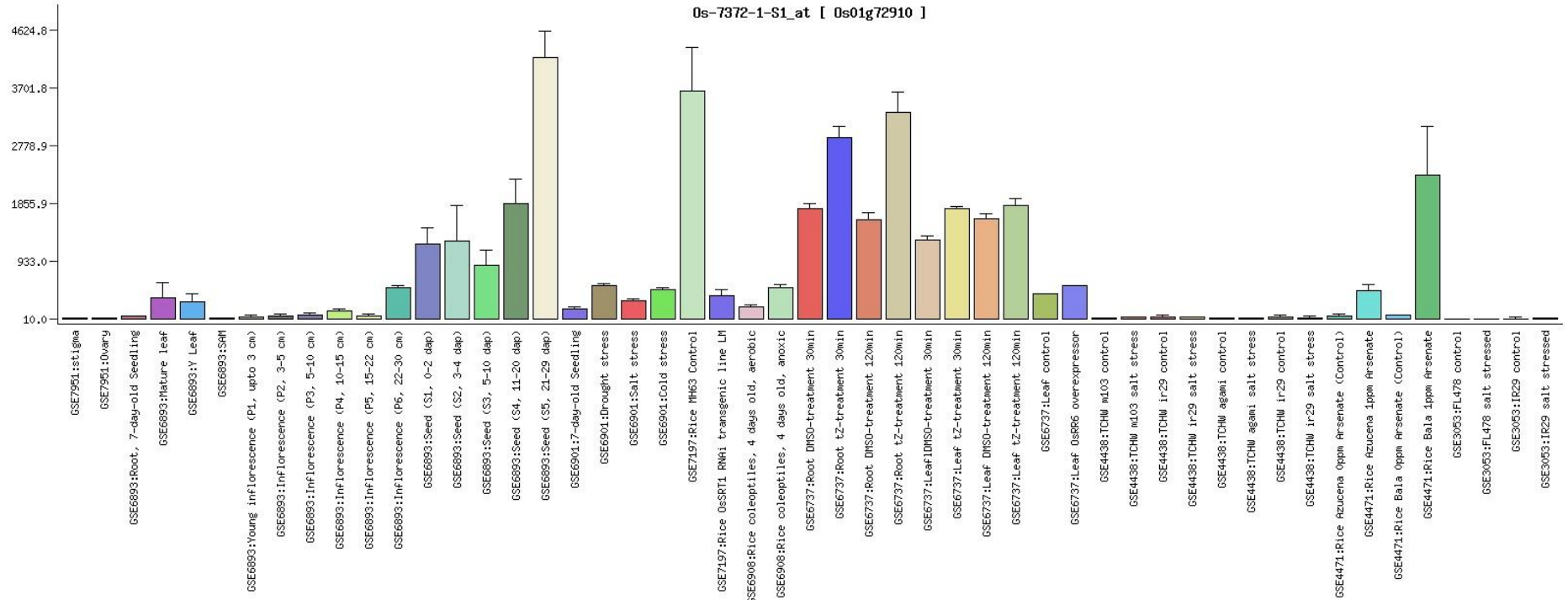

Figure 7. RiceGE: Gene expression atlas data sources.

heading stage, so tissue in these stages are hypersensitive to low temperature. Under heat treatment, the expression level of *OsASR* increased 14.13- and 31.72-fold in the leaf of booting and heading stage, but in the panicle of heading stage, the expression level changed slightly only 3.12-fold, so leaf of booting and heading stage are hypersensitive to high temperature. Under drought conditions, the expression level of *OsASR* upregulate slightly in the leaf of booting stage, but down-regulate in the panicle of booting and heading stage, so *OsASR* may be insensitive to water-deficient.

The expression profile of *OsASR* obtained by the microarray analysis was verified by quantitative real-time RT-PCR (Figure 6). The qRT-PCR result showed that expression levels of *OsASR* were increased at low temperature and high temperature, the expression pattern was generally similar to that revealed by the microarray analysis, suggesting that *OsASR* 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.

DISSCUSSION

We cloned the cDNA sequence of *OsASR* and analyzed its promoter (Figure 2). The results show that six ABRE, one GARE-motif, two MBS (involved in drought-inducibility) and other cis-elements were found in the promoter region, indicated that the expression of *OsASR* could be induced by abscisic acid, gibberellin, stress, ripening and so on. Many drought and high

salinity-inducible genes respond to ABA in *Arabidopsis* and rice (Seki et al., 2002; Rabbani et al., 2003). Most ABA-inducible genes contain aconserved, ABA-responsive, cis-acting element, designated as ABRE (PyACGTGG/TC) in their promoter regions. ABRE is a major cis-acting element in ABA-responsive genes (Nakashima et al., 2009). In *Arabidopsis*, the DRE/ CRTsequence may serve as a coupling element of ABRE in response to ABA (Narusaka et al., 2003), suggesting that the existence of the interaction between the DREB regulons and the ABRE-related regulons (Nakashima et al., 2009). *OsAsr1* gene play an important role during temperature stress, and this gene can be used for generating plants with enhanced cold tolerance (Kim et al., 2009). Based on the fact that *OsASR* responds to many kinds of stress conditions especially its high expression to short time cold treatment and heat treatment, several cis-elements were find in its promoter region and its deduced amino acid according to ORF was not big, we can take *OsASR* as an important candidate gene for abiotic stress tolerance.

Analysis with BALSTp of *OsASR* gene showed that it is similar to ASR protein (Figures 4 and 5). Genes encoding the ASR protein have been identified in several plant species, such as tomato (Iusem et al., 1993), maize (Riccardi et al., 1998), lily (Wang et al., 1998) and rice (Vaidyanathan et al., 1999; Kim et al., 2009). The amino acid sequences of ASR proteins in these species are hydrophilic and structurally related to each another (Frankel et al., 2006).

The GeneChip rice genome array and quantitative real-time RT-PCR analysis showed that *OsASR* was highly expressed in the leaves of seeding, booting and heading stage after shot time cold treatment and heat treatment, also slightly induced by drought conditions in booting stage leaf and heading stage panicle (Figure 6). The average upregulation in the leaf under cold treatment with GeneChip rice genome array and real-time quantitative PCR was 15.12- and 18.11-fold, and under heat treatment, the average upregulation was 22.92- and 20.42-fold, respectively. Researches find that ASR genes seem to be involved in processes of plant development, such as senescence and fruit development, and in responses to abiotic stresses, such as water deficit, salt, cold, and limited light (Schneider et al., 1997; de Vienne et al., 1999; Maskin et al., 2001; Jeanneau et al., 2002). In different species, ASR genes are expressed in various organs, such as the fruit of tomato, pomelo, and apricot (Iusem et al., 1993; Canel et al., 1995; Mbeguie-A-Mbeguie et al., 1997), the roots and leaves of tomato, rice and maize (Amitai-Zeigerson et al., 1994; Riccardi et al., 1998; Vaidyanathan et al., 1999), the tubers of potato (Silhavy et al., 1995), and the pollen of lily (Wang et al., 1998). Thus, distinct members of one *ASR* family may be expressed in different organs, under different conditions, and with different expression patterns (Canel et al., 1995; Maskin et al., 2001). Based on the restriction of conditions, we cannot 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 *OsASR*, 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 *OsASR* (Os.7372.1.S1_at). The columns in the black rectangle were expression data for stress treatment in rice seedlings of *O. sativa*. The results show that the expression level of *OsASR* was upregulated obviously under cold stress treatment, which was in coincidence with the results of GeneChip rice genome array (Affymetrix) and real-time quantitative PCR (Figure 7).

This work describes the identification and molecular characterization of a new temperature regulated gene *OsASR* from Pei'ai64s, whose expression in leaves of seeding, booting and flowering stage was upregulated obviously after cold and heat treatment. The complete cDNA is 379 bp in length and was cloned from Pei'ai64s. The longest ORF within encodes a protein of 105 amino acids with a calculated molecular mass of 11.7kD and an isoelectric point of 10.4. The homology genes were found in rice, *Hordeum vulgare*, *Sorghum bicolor*, *Zea mays*, *Solanum chilense* and so on. They are similar to abscisic acid stress ripening protein and involved in the abiotic stress tolerance. Seven (7) kinds of cis-elements related to stress responses were found in the predicted promoter region, further certified that *OsASR* is related to stress tolerance.

ACKNOWLEDGEMENTS

This research was supported by Fund 11ZA090 (A Project Supported by Scientific Research Fund of Sichuan Provincial Education Department) to RJ Chen, We thank Dr XJ Xia for array technique instruction.

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