OsSLI1, a Homeodomain Containing Transcription Activator, Involves Abscisic Acid Related Stress Response in Rice (Oryza sativa L.)

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Homeodomain-leucine zipper type I (HD-Zip I) proteins are involved in the regulation of plant development and response to environmental stresses. In this study, OsSLI1 (Oryza sativa stress largely induced 1), encoding a member of the HD-Zip I subfamily, was isolated from rice. The expression of OsSLI1 was dramatically induced by multiple abiotic stresses and exogenous abscisic acid (ABA). In silico sequence analysis discovered several cis-acting elements including multiple ABREs (ABA-responsive element binding factors) in the upstream promoter region of OsSLI1. The OsSLI1-GFP fusion protein was localized in the nucleus of rice protoplast cells and the transcriptional activity of OsSLI1 was confirmed by the yeast hybrid system. Further, it was found that OsSLI1 expression was enhanced in an ABI5-Like1 (ABL1) deficiency rice mutant abl1 under stress conditions, suggesting that ABL1 probably negatively regulates OsSLI1 gene expression. Moreover, it was found that OsSLI1 was regulated in panicle development. Taken together, OsSLI1 may be a transcriptional activator regulating stress-responsive gene expression and panicle development in rice.

1. Introduction

Transcription factors can bind to specific cis-elements in gene promoter regions. Through the activation or inhibition effects on gene expression, they play important roles in regulation of plant growth and development and morphogenesis, as well as responses to environment stimuli [1]. To date, the database of rice transcription factors (DRTF) contains 2,025 putative transcription factors (TF) in Oryza sativa L. ssp. indica and 2,384 in Oryza sativa L. ssp. japonica, distributed in 63 families [2]. The main transcription factor families among them (over 100 family members in indica/japonica) are AP2/EREBP (174/182), bHLH (157/184), bZIP (88/109), C2H2 (94/113), HB (84/103), MYB (136/138), NAC (157/149), and WRKY (I11/113).

The Homeodomain-leucine zipper (HD-Zip) genes have diverse functions in plant development and have often been implicated in stress adaptation [3]. The HD-Zip genes are the most abundant group of homeobox (HB) genes in plants and do not exist in other eukaryotes [4]. A typical DNA binding domain of homeobox proteins contains 60 amino acids, known as homeodomain. The homeodomain folds into a characteristic 3D structure containing three alpha-helices, of which the second and third form helix-turn-helix motif recognize and interact with specific DNA sequences as homodimers [5]. Agalou et al. identified 33 HD-Zip genes in rice and classified them into family I (14 genes), family II (14 genes), and family III (5 genes) [6]. The HD-Zip IV family in other higher plants has been previously described [7, 8].

Söderman et al. first identified an HD protein ATHB-7 from Arabidopsis thaliana, which was highly induced by salt, osmotic stress, and ABA treatment [9]. Later, Leead Chun identified Athb-12, which shares over 80% identity with ATHB-7 in their homeodomain and Zip motifs [10]. Expression of Athb-12 was induced by water stress and exogenous abscisic acid (ABA). Another HD-Zip gene, ATHB6, was expressed constitutively in seedlings but significantly upregulated in seedlings subjected to water deficit, osmotic...
stress, or exogenous treatment with ABA [11]. Athb-12 was also isolated by functional complementation of the NaCl-sensitive phenotype of a calcineurin (CaN-) deficient yeast mutant and it could increase NaCl tolerance but not osmotic stress tolerance of these mutant cells if expressed [12]. Zhu et al. isolated an Arabidopsis mutant hos9-1 and suggested that HOS9 mediates cold tolerance through a CBF-independent pathway [13].

Beside the model plant Arabidopsis, a number of HD-Zip proteins involved in abiotic stress response were also identified in the last decade from diverse crop plants. An HD-Zip protein was identified closely related to low temperature stress in hot pepper (Capsicum annuum L.) [14]. HAHB4 belongs to the sunflower HD-Zip proteins subfamily I and is involved in drought response and ethylene-mediated senescence [15]. In cotton, the GhHBI was isolated from a cotton root cDNA library. The expression level of the GhHBI gene was dramatically increased in roots under treatment with 1% NaCl and exogenous abscisic acid [16]. Expression of the cotton root cDNA library. The expression level of the GhHBI gene was dramatically increased in roots under treatment with 1% NaCl and exogenous abscisic acid [16]. Expression of Nicotiana attenuata NaHD20 was induced by multiple stress-associated stimuli including drought and wounding. It was predicted to be involved in leaf ABA accumulation during water stress, benzylacetone emission from flowers, and the timing of bolting andflower transitions [17]. Several drought-related members of the HD-Zip gene family were also characterized [6].

Rice is one of the most important food crops in the world, feeding half of the world’s population. Salt and drought are two major abiotic stresses for agricultural production [18]. With a relatively small genome size, rice is an ideal model plant for molecular and genetic research. It is critical to identify the roles of stress-induced genes in order to develop transgenic crops that have enhanced tolerance to unfavorable growth conditions or help breeding programs by molecular assistant selection [19].

In the present study, OsSLII encoding a member of HD-Zip I subfamily was analyzed. Expression of OsSLII was regulated by panicle development and dramatically induced by salt, drought, and ABA treatment. An ABI5-like transcription factor ABL1 probably negatively regulates OsSLII gene expression in rice under stress conditions. OsSLII may be a transcriptional activator regulating stress-responsive gene expression and panicle development in rice.

2. Materials and Methods

2.1. Plant Materials and Stress Treatments. The seeds of rice (Oryza sativa L. sub. japonica) cultivar Jiucaiqing, the rice ABI5-Like1 (ABL1) deficiency mutant, abl1, and its wild type Zhonghua 11 (ZH11, japonica) were sterilized in 0.1% HgCl2 and germinated at 30°C. The seedlings were cultured with Yoshida’s culture solution in growth chamber as previously described [20]. The 2-week-old seedlings were used for subsequent stress treatments. For H2O2, salt, PEG, and ABA treatments, seedlings were transferred to nutrients solutions containing 100 mM H2O2, 100 mM NaCl, 20% (w/v) PEG6000, and 50 mM ABA, respectively. For cold and heat treatments, seedlings were transferred to the growth chamber with the temperature of 4°C and 42°C, respectively. For drought treatment, germinated seeds were planted in 15 cm diameter pots filled with soil, watered daily for 2 weeks; watering was then withheld to simulate a natural dehydration environment. All samples were collected at various times after treatments, immediately freezed in liquid nitrogen, and stored in −80°C freezer.

2.2. RNA Isolation and First Strand cDNA Synthesis. Total RNA was extracted from various stress-treated rice seedlings using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. The RNA was subsequently treated with DNase I (Promega, USA) to remove the remaining genomic DNA. The first strand cDNA was synthesized with 2 μg total RNA using the reverse transcription system (Promega, USA).

2.3. Cloning of OsSLII. Pairs of primers (forward: 5’-TAGTGGCTCTGGTCTTACATT-3’ and reverse: 5’-TACGCGTATTGAGATAGCATG-3’) were employed to clone OsSLII by RT-PCR approach. The PCR conditions for amplifying OsSLII were as follows: 5 min predenaturation at 95°C, 32 cycles of 20 s at 95°C, 20 s at 56°C, and 50 s at 72°C, and a final extension for 5 min at 72°C. The PCR product was purified and cloned into pMD19-T vector (Takara, Japan) for sequencing.

2.4. Quantitative RT-PCR Assay. qRT-PCRs were carried out on an Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems). The qRT-PCR mixture of 20 μL was prepared using the FastStart Universal SYBR Green Master (Roxy) (Roche Diagnostics). Quantification of gene expression was done using the comparative CT method. Experiments were performed in triplicate and the results were represented by means ± SE of three replicates. 18S rRNA was used as an internal control [21]. The primers of 18S rRNA were as follows: forward primer: 5’-ATGGTGTTGACGGGTGAC-3’; reverse primer: 5’-CAGACACTAAGCGCCTGGTA-3’.

2.5. Multiple Sequence Alignment and Phylogenetic Analysis. Alignment of rice or other plant homeodomain protein sequences were performed with ClustalX program (ver 2.1). The phylogenetic tree was constructed with MEGA program (ver 4.0) by neighbor-joining (NJ) method. The parameters pairwise deletion and p-distance model were used. Bootstrap test of phylogeny was performed with 1,000 replicates.

2.6. Subcellular Localization. A green fluorescent protein (GFP) fusion protein was constructed using the full-length OsSLII cDNA clone with a C-terminal fusion of the GFP clone under the control of CaMV 35S promoter. Rice protoplast preparation and transformation were performed as previously described [22]. The subcellular distribution of the GFP fusion protein was examined by confocal laser scanning microscopy (Leica, TCS SP2). Cells were labeled with the DNA dye 4,6-diamidino-2-phenylindole (DAPI) to visualize the nucleus.
2.7. Transcriptional Activation Assay. The transcriptional activation activity of OsSLII was determined as previously described [23]. The full-length ORF of OsSLII was cloned into the pGBK7 vector containing the GAL4 DNA binding domain to obtain pGBK7-OsSLII. According to the protocol of the manufacturer (Stratagene, USA), pGBK7-OsSLII and the negative control pGBK7 vector were transferred into the yeast strain AH109, respectively. The transformed yeast cells were grown on SD glucose medium plates lacking Trp. Single colony (2 mm diameter) was picked and incubated in SD glucose liquid medium lacking Trp at 30°C for 2-3 days. Proper volume of the culture was transferred on SD glucose medium plate with 5 µM 3-AT and lacking Ade and His and incubated at 30°C for 2-3 days. The transactivation activity of each protein was evaluated according to its growth status and β-galactosidase assay (Yeast Protocols Handbook, Clontech).

2.8. In Silico Sequence Analysis. For promoter sequence analysis, the 2,000-bp upstream sequence of OsSLII was analyzed by Matinspector program at Genomatix website (http://www.genomatix.de/). The coexpression analysis was performed both with Rice Oligo Array Database (http://ricearray.org/) based on 1,081 rice Affymetrix microarray data (NCBI GEO AC: GPL2025) and by genevestigator (https://www.genevestigator.com/gv/). The coexpressed genes with OsSLII were selected with a Pearson’s correlation coefficient (PCC) cutoff of 0.6 in both results. The expression changes of coexpressed genes under cold, salt, and drought stresses were investigated using GEO (Gene Expression Omnibus) datasets. For promoter analysis, the 1,000-bp upstream sequences of each coexpressed gene were analyzed by our in-home program.

3. Results and Discussion

3.1. OsSLII Belongs to HD-Zip Family. Through a microarray approach (data not shown), an EST probe (ID: Os.49245.1.S1) was found with high induction levels in two-week-old rice seedlings by 100 mM NaCl (227-fold induction), and 20% (w/v) PEG6000 (134.5-fold induction). This EST was chosen for further research due to its dramatic expression increase under abiotic stress treatments. Database searching showed that this EST sequence encodes a putative homeodomain containing protein. Here, we named this gene *OsSLII* stress largely induced 1 as its expression is largely induced by multiple abiotic stresses and ABA treatment.

To elucidate the function of OsSLII, the full-length OsSLII cDNA was amplified from rice by RT-PCR. OsSLII is located on chromosome 2. The 783 bp ORF was predicted to produce a 261 amino acids protein with the calculated molecular mass of 28.54 kDa and the PI of 4.61. Putative conserved domains were detected through BLAST program of NCBI, including a 56-AA HD domain (65–121) for DNA binding and a 43-AA Zip domain (122–164) for protein-protein interactions. Previously, Agalou et al. investigated HD-Zip gene family in rice, confirmed 31 expressed genes of the total 33 family members, and assigned 14 of them, including OsSLII into HD-Zip I subfamily [6].

Multiple stress-related HD-Zip protein sequences were collected to perform multiple sequence alignment and phylogenetic analysis (Figures 1 and 2). Sequence alignment showed that HD domain and Zip domain are highly conserved among all the 12 proteins. The phylogenetic analysis revealed that OsSLII was highly close to MtHB2 and Oshox22. Previous study suggests that MtHB2, which were identified from *Medicago truncatula*, may play a negative role in regulation of abiotic stress response in *Arabidopsis* [24]. However, Oshox22 affects ABA biosynthesis and regulates drought and salt responses through ABA-mediated signal transduction pathways with a positive role [25]. These results indicate that HD-ZIP proteins may play different roles in stress responses in plants.

3.2. Expression of OsSLII in Different Rice Tissues. For tissue specific expression analysis, root and shoot tissues from two-week-old seedlings were collected. Booting stage leaves (Leaf), leave sheath (LS), and panicles with the lengths of 3 cm (P1), 8 cm (P2), and 12 cm (P3) were also collected from unstressed field grown plants. Quantitative RT-PCR result showed that, in the seedlings, OsSLII expression was mainly in root. The root tissue accumulates 7 times of OsSLII transcripts than in shoot tissue. In panicles, the expression changes in different development stages, from relative low in 3 cm young panicles to a 33-fold increase in 8 cm panicles, and the 12 cm panicles still accumulate 8-fold OsSLII transcripts compared to 3 cm young panicles tissue (Figure 3).

It is interesting that OsSLII transcript accumulates much more in reproduction organs compared to other tissues in rice. In *Arabidopsis*, transgenic lines overexpressing sunflower HAHB4 gene, a compact inflorescence, and short pedicels were observed [26]. Under control growth conditions, NaHD20-silenced plants presented a delay in bolting compared with empty vector control plants. The production of the first opened buds was delayed in NaHD20-silenced plants; the number was also significantly reduced [17]. Considering that HAHB4, NaHD20, and OsSLII were all classified into HD-Zip subfamily I, it is of interest to investigate whether members of this subfamily also are involved in reproduction organ development in plants.

3.3. Expression of OsSLII under Different Abiotic Stress Conditions. Since microarray data showed that OsSLII was largely induced by drought and salt stresses and ABA treatment, we investigated the expression pattern of the gene in response to various abiotic stresses. The rice cultivar Jiucaiqing, which was tolerant to drought and salt stresses [27,28], was used in abiotic stress treatment.

Quantitative RT-PCR assay showed that OsSLII expression was highly induced by salt, PEG, H₂O₂, high temperature, and ABA treatment but not by cold treatment. For salt treatment, OsSLII reached a 159-fold expression peak after a 3-hour treatment and maintained high expression till 48 hours. For PEG treatment, OsSLII transcript was slowly accumulated, starting responsiveness to the treatment after 6
4. cis-Acting Element Analysis. The upstream 2,000 bp DNA sequence of OsSLII was used for cis-acting element analysis. Some putative stress-related cis-elements, such as ABRE (ABA-responsive element binding factors), CGCG (calmodulin binding/CGCG box binding proteins), CRT/DREB (dehydration responsive element binding factors), GBOX (plant G-box/C-box bZIP proteins), MYBL (MYB-like proteins), and NACF (plant specific NAC transcription factors), were found (Figure 5). A total of 15 ABREs were found in this region 13 of which are located within the upstream 1,000 bp, indicating that the expression of OsSLII might be tightly regulated by ABF/bZIP type transcription factors. These stress-related cis-elements may be responsive for stress-responsive expression of OsSLII.

Figure 1: Sequence alignment of stress-related HD-Zip proteins in plants. The HD domain and Zip domains are boxed in black and red, respectively. GenBank accession numbers for the sequences: AtHB6 (AT2G22430), AtHB7 (AT2G46680), AtHB12 (AT3G61890), GhHB1 (EF151309), Hahb1 (AAA63765), Hahb4 (AAA63768), MtHB1 (ABO47743), MtHB2 (ACJ84212), NaHD20 (HM107874), Oshox1 (AAG13598), Oshox22 (AAO72559), and OsSLI1 (NP_001047582).

Hours, and reached the peak at 24 hours. OsSLII expression profiles under H2O2 and high temperature treatment were very similar, showing a two-peak induction pattern at 20 min and a 3-hour treatment with an expression decrease in 1-hour treatment. 50 μM exogenous ABA was applied to the seedlings as a signal molecule. OsSLII expression was induced by 907-fold after 12 hours of induction and then decreased to 78-fold till 48 hours. For low temperature treatment, OsSLII expression changes were not dramatic compared to other stresses (Figure 4). For drought, OsSLII expression was induced 4 times at 36 hours as compared to unstressed samples and reached its peak of 170-fold induction at 60 hours; then the expression decreased slowly and still maintained an 85-fold expression level at 84 hours.
Table 1: List of genes coexpressed with OsSLI1.

| Gene ID          | Annotation                                         | PCC1   | PCC2   | Drought | Salt | Cold |
|------------------|----------------------------------------------------|--------|--------|---------|------|------|
| LOC_Os09g15670   | Protein phosphatase 2C                              | 0.7719 | 0.64   | 7.16    | 6.29 | 4.27 |
| LOC_Os03g16170   | Protein phosphatase 2C                              | 0.6799 | 0.68   | 5.69    | 4.44 | 3.64 |
| LOC_Os07g05940   | 9-cis-Epoxycarotenoid dioxygenase, chloroplast precursor | 0.6140 | 0.70   | 6.92    | 5.93 | 6.45 |
| LOC_Os07g48830   | Glycosyltransferase 8 domain containing protein     | 0.6805 | 0.63   | 5.07    | 3.97 | 3.78 |
| LOC_Os06g41360   | Phosphoribosyltransferase                           | 0.6511 | 0.62   | 1.98    | 1.21 | −0.42|
| LOC_Os05g41490   | Circadian clock coupling factor ZGT                 | 0.6115 | 0.61   | 6.45    | 5.88 | 3.01 |

PCC: Pearson’s correlation coefficient. 1: ricearray; 2: genevestigator. The data in the table indicate the log2-fold of the microarray data in rice seedlings under drought, salt, or cold stress relative to that under control condition. The normalized microarray data were obtained from GEO database under accession number GSE6901.

Yeast hybrid system was employed to investigate the transcriptional activity of OsSLI1. As shown in Figure 7, all transformants grew well on SD/Trp medium. However, only transformants containing pGBK7-OsSLI1 could grow on SD/Trp-/His-/5 mM 3-AT medium, whereas the yeast cells transformed with pGDB7 control failed to grow. These results were consistent with the β-galactosidase activity analysis, and indicated that OsSLI1 had transactivation activity.

3.6. Expression of OsSLI1 in Rice abl1 Mutant under Abiotic Stress. The rice ABI5-Like (ABL1) deficiency mutant, abl1, shows suppressed ABA responses [29]. Since evidences from previous data suggest OsSLI1’s expression pattern is ABA related, it is interesting to analyze whether OsSLI1 is involved in ABL1 signaling. We analyzed OsSLI1 gene expression in abl1 and wild-type rice ZH11 under salt and drought stresses. After 12 hour of induction by PEG, OsSLI1 gene expression increased to 4,515-folds compared to untreated samples in abl1, while in WT its expression only increased 51 times. For NaCl treatment, 12 hours of induction in abl1 showed 4,900 times higher than untreated samples while, in WT, it was only induced 1.6 times. Similarly, OsSLI1 expression was 302- and 33-fold increased by ABA treatment in abl1 and WT, respectively. OsSLI1 expression was enhanced in abl1 mutant under both normal and stress conditions (Figure 8), suggesting that ABL1 probably negatively regulates OsSLI1 gene expression.

3.7. Coexpression Analysis. A total of 96 positively coexpressed genes and one negatively coexpressed gene were selected with Pearson’s correlation coefficient higher than 0.6 from the OsSLI1 coexpression gene data by rice array database. Coexpression analysis by Genevestigator discovered 35 genes positively coexpressed with OsSLI1. Six genes were found in both results (Table 1). Five of the six genes showed upregulated response to all three abiotic stresses. Only LOC_Os06g41360, a phosphoribosyltransferase, was slightly induced by drought and salt treatments and inhibited by cold stress. For the other five genes, one gene (LOC_Os07g05940) encodes NCED, a key enzyme in ABA biosynthesis pathway showed upregulation by cold. The top 2 genes with a higher PCC value encode protein phosphatase 2C, which negatively regulate ABA signaling at an early step in the pathway [30]. The result of coexpression analysis

3.5. Subcellular Localization and Transcriptional Activation Analysis. To control downstream gene expression, most transcription factors locates in the nucleus. A pCaMV 35S:OsSLI1-GFP plasmid was constructed and introduced into rice protoplasts through PEG mediated transient transformation. The fusion protein was detected in rice protoplast nucleus part, which was indicated by DAPI staining. This observation suggests that OsSLI1 is a nuclear-localized protein, in consistency with its potential function as a DNA binding transcription factor (Figure 6).
Figure 4: Expression change of OsSLII in rice seedlings under different treatments. (a) salt stress (100 mM NaCl); (b) osmotic stress (20% PEG6000); (c) ABA treatment (50 μM ABA); (d) oxidative stress (100 mM H₂O₂); (e) high temperature (42°C); (f) low temperature (4°C); and (g) drought treatment.
The plant-specific homeodomain-leucine zipper type I (HD-ZIP I) proteins play important roles in plant development and stress responses. In this research, OsSLI encoding an HD-ZIP I protein was isolated from rice. Time-course expression analysis indicated that OsSLI expression was induced very quickly and dramatically by various abiotic stresses including salt, drought, osmotic treatment, and exogenous abscisic acid. The promoter sequence of OsSLI harbors several cis-elements including high density of ABA-responsive elements, but the well-known CRT/DREB element is much less. Further, it was found that OsSLI expression was enhanced in an ABI5-Like1 (ABL1) deficiency mutant abl1 under both normal and stress conditions, suggesting that ABL1 probably negatively regulates OsSLI gene expression. It was found that OsSLI was also regulated in panicle development. The OsSLII-GFP fusion protein was localized in the nucleus of rice protoplast cell. Yeast hybrid assays revealed that OsSLII had transcription activation activity, indicating that OsSLII functions as a transcription activator. Taken together, OsSLII may be a transcriptional activator regulating stress-responsive gene expression and panicle development in rice. Further studies with OsSLII transgenic plants will be necessary to elucidate the roles of OsSLII in stress responses and panicle development in rice.
Conflict of Interests
The authors declare no conflict of interests.

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