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OsGSK3 IS A NOVEL *GSK3/SHAGGY*-LIKE GENE FROM *ORYZA SATIVA* L., INVOLVED IN ABIOTIC STRESS SIGNALING

XIAN-GUANG YANG[1], WEI-HONG LIANG[1], FEN LI[1] AND WEN-SHI MA[2*]

[1]*School of Life Sciences, Henan Normal University, Henan, Xinxiang 453007, P. R. China*
[2]*School of Chemistry technology, Shijiazhuang University, Hebei, Shijiazhuang 050001, P. R. China*
[*]*Corresponding author's e-mail: yangxg@htu.cn*

Abstract

Plant glycogen synthase kinase3/shaggy-like (GSK-3-like) kinases are produced by a multigene family. Here, a novel full-length cDNA encoding rice glycogen synthase kinase3/shaggy-like (GSK-3-like) kinase was cloned for the first time using reverse transcription polymerase chain reaction (RT-PCR). In order to investigate the genomic organization of *OsGSK3*, southern hybridization was done using *OsGSK3* cDNA as the probe, and genome DNA were digested by *Eco*R I and *Hind*III restriction enzyme separately. Results illustrated that there was only one copy of *OsGSK3* in the rice genome. Studies evaluating changes in the plants over time showed that the accumulation of *OsGSK3* in the rice cultivars Nipponbare (*Oryza sativa L.subsp. japonica*) was increased by salt stress (170 mM NaCl), mechanical injury, ABA hormone, cold (4°C) and drought. These results suggest that cell accumulate more *OsGSK3* mRNA response to those abiotic stresses. This suggests that enhanced *OsGSK3* expression may be related to abiotic stress response, and may play an important role in the stress signal transduction. In this way, OsGSK3 was shown to be a positive regulator and to have some part in rice tolerance to salt, mechanical injury, ABA hormone, cold, and drought.

Introduction

Abiotic stresses are serious threats to crop yields and they cause considerable damage to natural environments. Soil salinity and drought are two major abiotic stresses in plant agriculture worldwide (Mahajan & Tuteja, 2005; Balal *et al*., 2011), and bring seriously loss in agriculture production and shortage of food supplies. These stresses can cut crop production in half (Wang *et al*., 2003; Jamil *et al*., 2010). Furthermore, salinity and drought are becoming serious problems in more and more areas. This can cause plants to undergo morphological, physiological, biochemical and molecular changes. Salinity, drought, extreme temperatures and oxidative stress are often interconnected, and their effects can be similar. For example, Salinity and drought both cause osmotic stress, which disrupts ion distribution in the cell (Zhu, 2001).

Oxidative stress is frequently seen alongside high temperatures, salinity, and drought, It can inactivation both functional and structural proteins (Miller *et al*., 2010). Therefore, these three environmental stresses often activate specific cell signaling pathways and cellular responses, increasing the concentration of stress proteins, up-regulating the production of anti-oxidants and triggering the accumulation of compatible solutes (Shi *et al*., 2010; Polidoros *et al*., 2009; Knight, 2001).Osmotic stress can activate mitogen-activated kinases, which can control the detoxification response. In addition, osmotic stress can activate a number of phospholipids systems, This activates a wide variety of messenger molecules, some of which mediate glycogen synthase kinase3/shaggy like kinase, and protein kinases activated by oxidative stress, (Zhu, 2002).

Animal glycogen synthase kinase (GSK) 3 can regulate certain physiological processes, incuding glycogen metabolism, protein synthesis, transcription factor activity, and developmental. However, little is known about the plant GSKs. Unlike mammals, which have 2 copies of GSK gene, plants have multigene families, such as the one that encodes plant GSKs. GSK-3-like genes have been cloned from several plants, such as *Arabidopsis* (Rozhon *et al*., 2010; Tavares *et al*., 2002; Dornelas *et al*., 1999; Jonak *et al*., 1995), *Alfalfa* (Kempa *et al*., 2007), *Petunia hybrida* (Decroocq-Ferrant *et al*., 1995), *Nicotiana tabacum* (Einzenberger *et al*., 1995), and *Triticum aestivum* (Chen *et al*., 2003). A series of previous studies has indicated that plant GSKs are involved flower development, wound responses, salt stress and brassinosteroid signaling (Dornelas *et al*., 2000; Jonak *et al*., 2000; Piao *et al*., 2001; Li *et al*., 2002). Here, we cloned and characterized a member of GSK3 family from rice plant (*Oryza sativa L.*), and the expression patterns of this GSK family member in response to salt, mechanical injury, ABA hormone, cold (4°C) and drought are reported.

Materials and Methods

Plant material and stress treatments: Seeds of the rice (*Oryza sativa* L. *sub sp. japonica*) cultivar Nipponbare were immersed in water at 30°C for 3 days. Germinated seeds were replanted in a flowerpot containing vermiculite. The seedlings were irrigated with distilled water. Two-week-old plants treated with distilled water only served as a control during Northern blot analysis. Experimental plants were exposed to high concentrations of salt (170 mM NaCl), mechanical injury (shearing), ABA hormone (100 μM), cold (4°C), and 30％PEG6000 (simulated drought). Plant were harvested at different time after stress treatments, frozen in liquid nitrogen and stored at -80°C before use.

RNA isolation and cDNA synthesis: Total RNA was isolated using a total RNA isolation system in according with the manufacturer's instructions (Shanghai Sangon). Oligo (dT_{18}) was used to synthesize single-strand cDNAs using the protocol included with the M-MLV Reverse Transcriptase kit (Promega). The resulting single-strand cDNA mixtures were used as templates for reverse transcriptase polymerase chain reaction (RT-PCR).

Isolation of OsGSK3 full-length cDNA: The primer pair P1 (5′-CTC CGA ATC CTC CCC CGC AT-3′) and P2 (5′-GGA CCG AAC GAA CAT TGC CA-3′) were based on conserved sequences from the known plant GSKs. Hot-start PCR was performed using the single-strand cDNA template and Ex-Taq polymerase (Takara) on a Biometra T-GRADIENT Themoblock using the following protocol: 30 cycles of 94°C for 30 s, 61°C for 45 s, 72°C for 90 s. The PCR product was subcloned into pGEM-T vector (Promega) and sequenced (Shanghai Sangon). The gene was compared to the *Oryza sativa* genome using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/Genome/PlantBlast.shtml; plant choices: *Oryza sativa* ssp.*japonica* WGS contigs). Bioinformatic analyses were performed using DNASTAR 5.0 software (DNASTAR, Inc., Madison, WI, U.S).

DNA extraction and Southern analysis: Genomic DNA was extracted from the leaves of two-week-old rice plants according to the method described by Sambrook and Russell (Sambrook & Russell, 2001). For Southern hybridization, genomic DNA was digested with *Eco*RI or *Hind* III for 16 h separately. It was then subjected to electrophoresis in 0.8% (w/v) agarose for 16 h at 4V cm^{-1} (10μg per lane). The DNA was subjected to depurination within the gel in 0.25M HCl for 30 min at room temperature, and then transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, Little Chalfont, UK). The DNA was incubated in 20xSSC and hybridized with the ^{32}P-labeled gene-specific probe for 16 h at 65°C. The nylon membranes were washed once in 0.1% SDS, 2xSSC for 15 min 65°C and twice in 0.1% SDS, 0.1xSSC for 15 min at 65°C, which is considered high-stringency conditions. They were then exposed to X-ray film.

Northern blot analysis: Total RNA (30μg) was detached by 1.2% formaldehyde-agarose gels according to the method of Sambrook and Russell (Sambrook & Russell, 2001) , and blotted onto Hybond-N+ nylon membrane, which was then hybridized with the ^{32}P-labeled gene-specific probe for 16 h at 65°C. The nylon membrane were washed once in 0.1% SDS, 2xSSC for 15 min 65°C and twice in 0.1% SDS, 0.1xSSC for 15 min at 65°C, and then exposed to X-ray film.

Results and Discussion

cDNA clone of the OsGSK3 gene: The *OsGSK3* cDNA was found to contain 1783 bp and to have an open reading frame of 1,236bp (start codon at 216 bp and stop codon at 1451 bp,) encoding a deduced protein of 411 amino acids (GenBank Accession No. DQ060684). The theoretical isoelectric point (PI) of the putative amino acid sequence is 8.58 and its molecular weight (MW) is 43.5 kDa. Comparing of the *OsGSK3* cDNA sequence and *Oryza sativa* ssp.*japonica* genomic database showed that the *OsGSK3* gene is located in the 4918-8567 region of the rice genome AACV01010829 (*Oryza sativa* (*japonica* cultivar-group) chromosome 5 Ctg010829).

Phylogenetic analysis of OsGSK3 and GSKs from other plants: The amino acid sequences of *OsGSK3* and GSKs from *Triticum aestivum* (AF525086), *Zea mays* (AY108486), *Trifolium repens* (X99100), *Nicotine tabacum* (X77763), *Medicago sativa* (X68409) and *Arabidopsis thaliana* (BT000132) are aligned. The putative amino acid sequence of *OsGSK3* was showed to be highly homologous to GSKs from *Triticum aestivum* (94.2%), *Zea mays* (95.5%), *Trifolium repens* (90.5%), *Nicotine tabacum* (83.4%), *Medicago sativa* (84.4%) and *Arabidopsis thaliana* (73.2%) (Fig. 2). Phylogenetic analysis of *OsGSK3* was shown in Fig. 1.

Southern analysis of *OsGSK3*: Southern analysis of genomic DNA digested separately with two different restriction enzymes, *Eco*R and *Hind*III, was performed to estimate the copy number of *OsGSK3* in the Nipponbare rice genome. As shown in Fig. 3, *OsGSK3* full-length probe (1,783 bp) and a high-stringency wash produced distinctive band patterns. Two hybridizing bands were observed per digestion, which was consistent with the location of the cutting site in the probe. This indicated that one copy of the *OsGSK3* sequence was present in the Nipponbare rice genome.

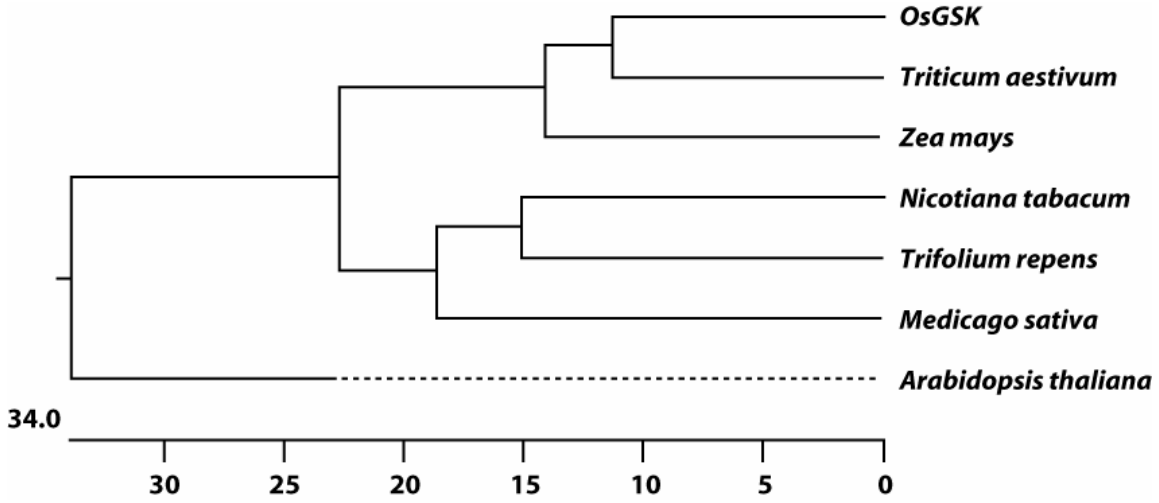


Fig. 1. Phylogenetic tree representing the relationship between OsGSK3 and other plant GSKs.

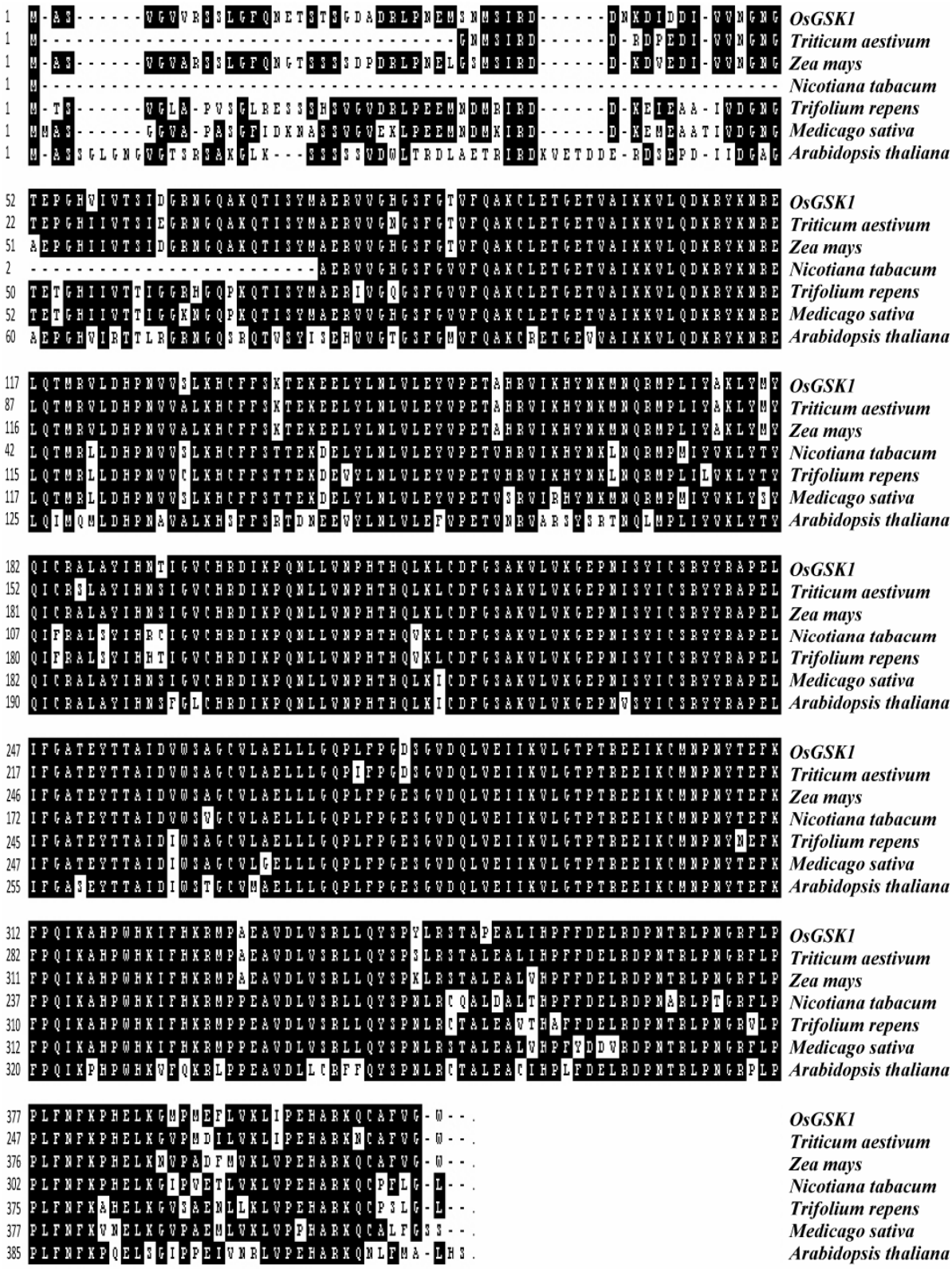


Fig. 2. Alignment of the deduced amino acid sequences of GSKs from *Oraza stiva* (GenBank DQ060684), *Triticum aestivum* (GenBank AF525086), *Zea mays* (GenBank AY108486), *Trifolium repens* (GenBank X99100), *Nicotiana tabacum* (GenBank X77763), *Medicago sativa* (GenBank X68409) and *Arabidopsis thaliana* (GenBank BT000132). The *black shading* indicates identical amino acids. Amino acid sequences were aligned using the MegAlign program (DNAstar software (DNASTAR, Inc., Madison, WI, USA)) and the CLUSTALW method.

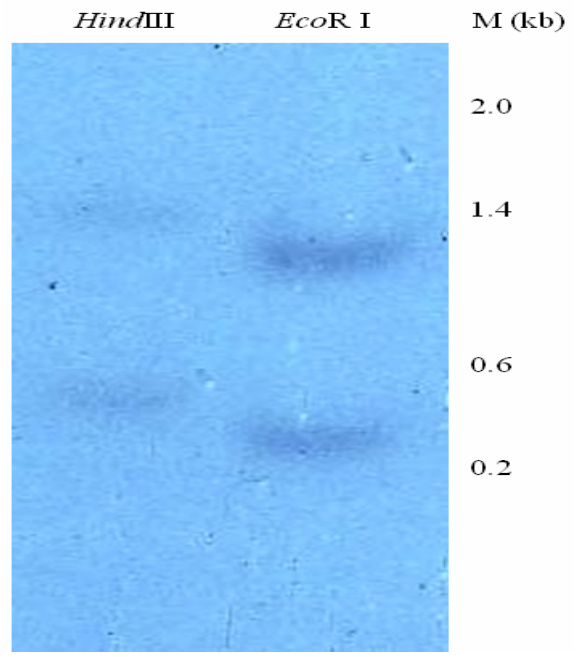
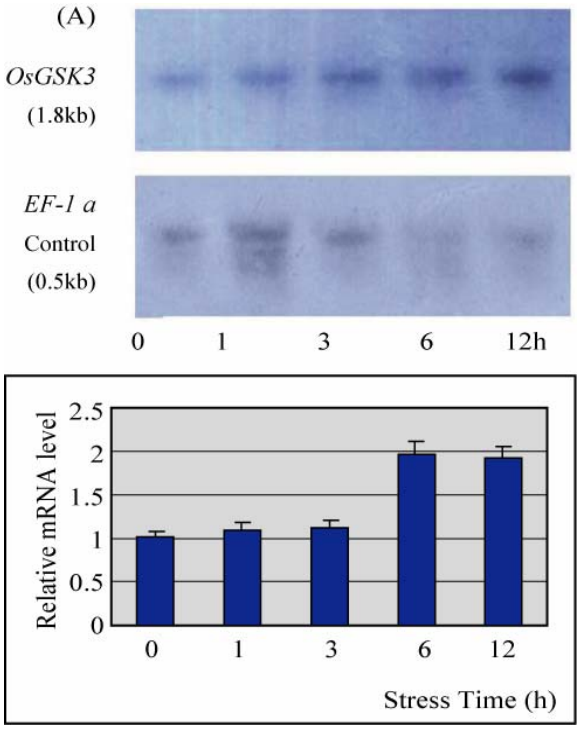


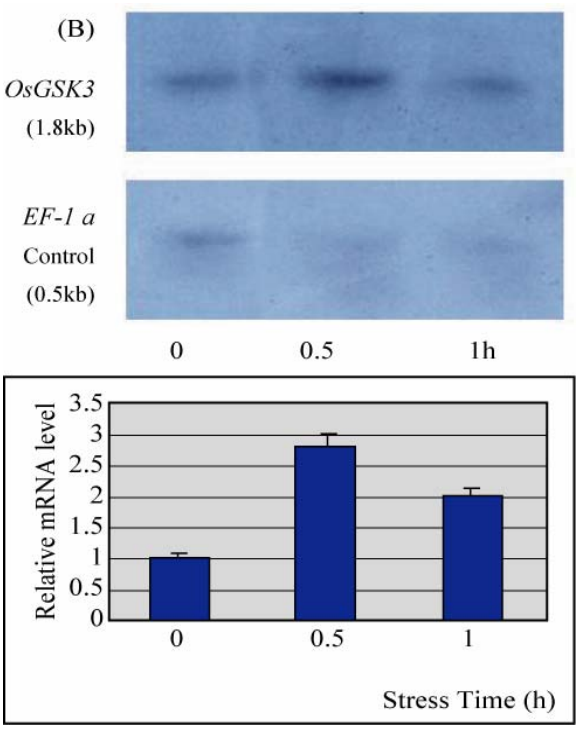
Fig. 3. Southern-blot analysis of *OsGSK3* Genomic DNA was digested with restriction enzymes and size separated onto a 0.8% agarose gel. The DNA was then transferred to a nylon membrane and cross-linked using heat. Hybridization was carried out with the *OsGSK3* cDNA overnight at 65°C.

Time-course of expression of the *OsGSK3* gene in response to abiotic stresses: The northern blot analysis of *OsGSK3* during abiotic stresses, such as salt stress (170mM NaCl), mechanical injury, ABA hormone treatment, drought and cold (4°C) were shown in Fig. 3. Constitutively expressed *EF-1α*was used as an internal control. As shown in Fig. 3, *OsGSK3* expression was upregulated under these abiotic stresses. Accumulation of *OsGSK3* mRNA increased step by step under salt stress (Fig. 4A). Its expression was up to 2 folds compared to the control when it was stressed 6 h with NaCl. Injury treatment had different effect to its expression, its expression increased rapidly, up to 3 folds when it was injured just half hour (Fig. 4B); its expression increased 1.6 folds after treated with ABA 1 h (Fig. 4C); 2.0 and 2.3 folds increase after treated with PEG6000 at different time, 6h and 12h (Fig. 4D), low temperature (4°C) can induce its high expression too (Fig. 4E).

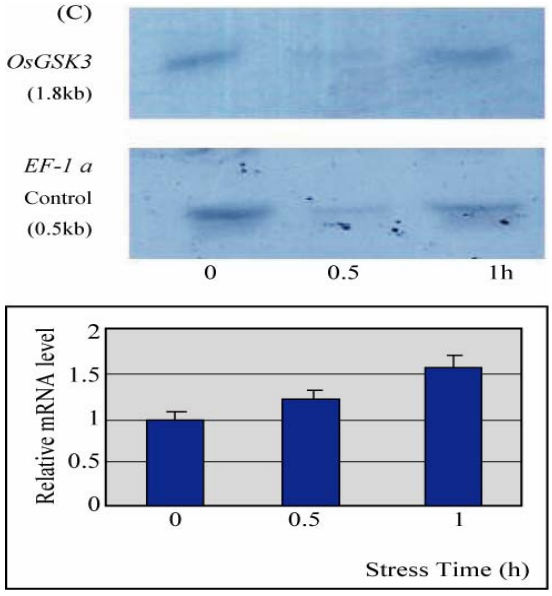
Glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases (GSKs) are non-receptor serine/threonine protein kinases. They are involved in many different biological processes. This is the first report of the RT-PCR cloning of *OsGSK3*, which is a member of rice GSKs family. By NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it belongs to the catalytic domain of protein kinases superfamily and contains the same catalytic domain as serine/threonine Kinases. The deduced polypeptide sequence of the full-length *OsGSK3* cDNA was found to share a great deal of identity with homologs from monocotyledons, including *Zea mays* (95.5%), *Triticum aestivum* (94.2%), *Trifolium repens* (90.5%), and a less higher identity with dicotyledons, including *Nicotine tabacum* (83.4%), *Medicago sativa* (84.4%) and *Arabidopsis thaliana* (73.2%). These results indicate that rice Glycogen Synthase Kinase3/shaggy like kinase gene was isolated successfully. Southern analysis indicated that only one copy of the *OsGSK3* gene is present in rice genome.



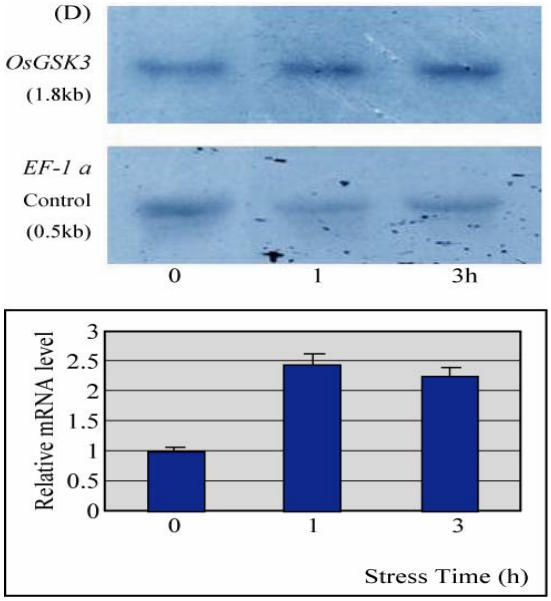
The relative mRNA level of *OsGSK3* under salt stress treatment



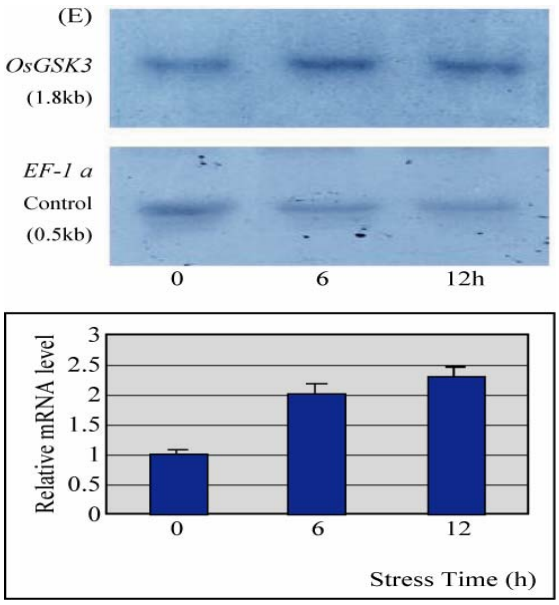
The relative mRNA level of *OsGSK3* under injury treatment



The relative mRNA level of *OsGSK3* under ABA treatment



The relative mRNA level of *OsGSK3* under cold treatment



The relative mRNA level of *OsGSK3* under drought treatment

Fig. 4. Northern blot analysis of *OsGSK3* gene expression in rice cultivar Nipponbare in response to salt stress (1%NaCl), mechanical injury, ABA hormone treatment, drought and cold (4°C). Using constitutively expressed *EF-1*α gene as an internal control. Two-week-old plant was treated with high concentration of (**A**) salt (170 mM NaCl), (**B**) mechanical injury (shearing), (**C**) ABA hormone (100 μm), (**D**) low temperature (4°C) and (**E**) drought (30%PEG6000), Harvest plant samples for total RNA isolation after stress treatment at certain time interval. Thirty micrograms of total RNA from each sample was loaded onto each lane and hybridized with ^{32}P-labeled full-length *OsGSK3*. Probe labeling using Random Primed DNA Labeling Kit (Takara). Relative levels of mRNA were analyzed by GENE GENIUS BIO IMAGING SYSTEM (USA) and MICROSOFT EXCEL software.

But, much less is known about the specific functions of these *GSK* genes in plants than animals. Present evidence indicates that plant GSKs are also involved in different processes, including development, hormone signaling, NaCl stress and wound responses. Consistent with these previous observations, we found in the present study that *OsGSK3* was also involved in response of rice abiotic stresses, such as salt stress, mechanical injury, cold, and simulated drought stress. And rice plants accumulate more *OsGSK3* mRNA response to all of these stresses and have stronger resistance to stress. The results presented provide evidence that the single copy *OsGSK3 gene* plays an important role in stress defense systems, and mediates more closely crosstalk among abiotic stresses. *OsGSK3* mRNA increases by 60% after 1 h of ABA hormone treatment, which suggest that *OsGSK3* genes not only contributes to various abiotic adaptations, but also participate in the growth and development, which was demonstrated for some stress-induced genes in both herbaceous and woody plants (Xiong *et al*., 2002; Cao *et al*., 2009). These means such stresses are interconnected and may induce similar cellular signal transduction pathways. Moreover, these signal pathways of abiotic stress response may cross-talked on *OsGSK3* or *OsGSK3* integrate all of these signal pathways in rice. At the first step, stresses signal activate the glycogen synthase kinase via different signal pathway. Secondly, some substrates involved in the stress response being phosphorylated and activity regulated. Finally, stress responsive genes express and start-up stress responsive mechanisms to re-establish cellular homeostasis and repair damaged proteins and membranes. In summary, OsGSK3 was shown to be a positive regulator involved in the tolerance to salt, mechanical injury, ABA hormone, cold, and simulated drought in rice. But, most mechanisms in this model are still unknown, and a clear link between OsGSK3 and the abiotic stress response has yet to be established.

Acknowledgments

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