

Full Length Research Paper

Molecular cloning of a novel *GSK3/shaggy*-like gene from *Triticum monococcum* L. and its expression in response to salt, drought and other abiotic stresses

Yang Xian-Guang, Fan Jin-Yu and Deng Chuan-Liang*

School of Life Science, Henan Normal University, Xinxiang 453007, China.

Accepted 25 March, 2011

The glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases are nonreceptor serine/threonine protein kinases that are involved in a variety of biological processes. Here, a novel GSK-3-like kinase encoding cDNA was isolated from *Triticum monococcum* L. seedlings by reverse transcriptase polymerase chain reaction (RT-PCR). Sequence analysis showed that the full length of cDNA consist of 1,543 bp with an open reading frame of 1,068 bp, which encodes 355 amino acid residues. The deduced amino acid sequence showed a high homology with shaggy-like kinases from *Triticum aestivum*, *Zea mays*, *Trifolium repens*, *Nicotine tabacum*, *Medicago sativa* and *Arabidopsis thaliana*; therefore, the gene was named *TmGSK1* (*T*riticum *m*onococcum *G*lycogen *S*ynthase *K*inase 1, GenBank Accession No. DQ443471). Southern blot analysis indicated that there was only one copy of *TmGSK1* in the einkorn wheat genome. Quantitative real-time RT-PCR studies showed that the expression of *TmGSK1* in the einkorn wheat was induced by salt stress, mechanical wounding, ABA hormone, cold and drought. These results suggest that cells accumulate more *TmGSK1* mRNA response to those abiotic stresses. *TmGSK1* was shown to be a positive regulator commonly involved in the tolerance to salt, mechanical injury, ABA hormone, cold and drought in einkorn wheat.

Key words: *TmGSK1*, abiotic stress, shaggy-like kinase, signal transduction, *Triticum monococcum* L.

INTRODUCTION

Abiotic stresses such as salinity, drought, extreme temperatures, chemical toxicity and oxidative stress are becoming particularly widespread in many regions. It is estimated that salinity affects at least 20% of the world's arable land and more than 40% of irrigated land to various degrees (Wang et al., 2003). These stresses reduce average yields for most major crop plants by more than 50%. Further more, drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may affect the plants in a similar way (Katerji et al., 2004; Almansouri et al., 2001). These diverse environmental stresses often activate similar cell signaling pathways and cellular responses such as the production of stress

proteins, up-regulation of anti-oxidants and accumulation of compatible solutes, leading to plant defense and/or adjustment to adverse conditions (Bajguz and Hayat, 2009; Zhu, 2002; Knight and Knight, 2001). Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic, and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress (Gill and Tuteja, 2010). Oxidative and osmotic stress activates several protein kinases including mitogen-activated protein kinases, which may mediate osmotic homeostasis and/or detoxification responses. A number of phospholipid systems are activated by osmotic stress, generating a diverse array of messenger molecules, some of which may function upstream of the osmotic stress-activated protein kinases (Zhu, 2001) and glycogen synthase kinase3/ shaggy like kinase.

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed protein kinase. It was originally identified as a regulator of glycogen synthesis in mammals. Animal GSK-3 participates in glycogen metabolism and in both the

*Corresponding author. E-mail: biol@htu.cn

Abbreviations: GSK-3, Glycogen synthase kinase-3; RT-PCR, reverse transcriptase polymerase chain reaction; PCR, polymerase chain reaction.

Wnt/ β -catenin and PI3K/AKT signaling pathways involved in the regulation of several physiological processes, including glycogen metabolism, protein synthesis, transcription factor activity and developmental control. Whereas, plant GSK-3-like kinases (GSKs) have only recently entered the scene. In contrast to mammals, which contain two genes, a multigene family encodes plant GSKs. GSK-3-like genes have been isolated from plants such as *alfalfa* (Pay et al., 1993), *arabidopsis* (Rozhon et al., 2010; Tavares et al., 2002; Piao et al., 1999; Dornelas et al., 1999; Jonak et al., 1995; Bianchi et al., 1994), *Petunia hybrida* (Decroocq-Ferrant et al., 1995), *Nicotiana tabacum* (Einzenberger et al., 1995), *Triticum aestivum* (Chen et al., 2003), *Oryza sativa* (Koh et al., 2007) and *Zea mays* (Zou et al., 2008). Present evidence indicates that plant GSKs are involved in different processes such as flower development (Dornelas et al., 2000), wound responses (Jonak et al., 2000), salt stress (Piao et al., 2001) and brassinosteroid signaling (Peng et al., 2010; Li and Nam, 2002). Einkorn wheat is a rare species that reduces the magnitude of population and the area of distribution under anthropogenic activity. It is closely related to cultivated wheat and has some properties, for example, resistance to diseases, and thus were used in selection to improve wheat varieties. Here, we isolated a member of GSK family from einkorn wheat and analyzed its expression patterns in response to abiotic stresses by real time quantitative polymerase chain reaction (PCR) approach.

MATERIALS AND METHODS

Plant material and stress treatments

Seed of the einkorn wheat (*Triticum monococcum* L.) were surface-sterilized for 5 min in 1% (w/v) sodium hypochlorite and was then washed in distilled water. The sterilized seeds were soaked in distilled water at 30°C overnight, for germination. The germinated seeds were transplanted into a pot containing vermiculite. The seedlings were irrigated with distilled water. Two-week-old plant was treated with distilled water (as a control for Q-PCR analysis), high concentration of salt (170 mM NaCl), mechanical injury (shearing), ABA hormone (100 μ M), cold (4°C) and drought (30%PEG6000). Plant was harvested after stress treatments at intervals of definite time, then immediately frozen in liquid nitrogen and stored at -80°C for later use.

RNA isolation and cDNA synthesis

Total RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. Total RNA was treated with RNase-free DNaseI (Promega) for 20 min at 37°C, DNase I was degraded at 65°C for 10 min. The integrity of the RNA was verified after separation by electrophoresis on a 0.8% agarose gel containing 0.5% (v/v) ethidium bromide. First-strand cDNA was synthesized from 500 ng of total RNA using Reverse Transcription system (Promega) with an oligo-dT₁₅ primer. The reaction solution was used as templates for reverse transcriptase polymerase chain reaction (RT-PCR).

TmGSK1 gene cloning and sequence analysis

PCR was performed using the following primers for *TmGSK1*: T1-forward (5'- GTTGGTGTGGTGCCTTC-3') and T1-reverse (5'- TGGGGGCCCTCGATCCATGAAC -3') designed from the conserved sequences of the known plant GSKs. Polymerase chain reaction (PCR) was initiated with hot start method using the single strand cDNA template and Ex-Taq polymerase (Takara) on Biometra T-GRADIENT Themoblock. The PCR reaction was carried out for 35 amplification cycles (94°C for 30 s, 61°C for 45 s and 72°C for 90 s). The RT-PCR product was cloned into pGEM-T vector (Promega) and sequenced. Bioinformatic analyses were carried out using DNASTAR software (DNASTAR, Inc., Madison, WI, USA).

DNA extraction and Southern analysis

Genomic DNA was isolated from green leaves of two-weeks-old einkorn wheat according to the method described in molecular cloning laboratory manual (Sambrook and Russell, 2001). For Southern hybridization, genomic DNA was digested separately with *EcoRI* and *Hind III* for 16 h and subjected to electrophoresis in 0.8% (w/v) agarose for 16 h at 4 V cm⁻¹ (20 μ g per lane). After depurination of DNA within the gel in 0.25 M HCl for 30 min at 25°C, the DNA was transferred to a Hybond-N+ nylon membrane (Amersham Bio- sciences, Little Chalfont, UK) in 20 \times SSC and hybridized with the probe, which was labeled with [α -³²P] dCTP by Random Primer DNA Labeling kit(Takara), for 16 h at 65°C. The blots were washed once in 0.1% SDS, 2 \times SSC for 15 min at 65°C and twice in 0.1% SDS, 0.1 \times SSC for 15 min at 65°C (high-stringency conditions), and exposed to X-ray film (Kodak).

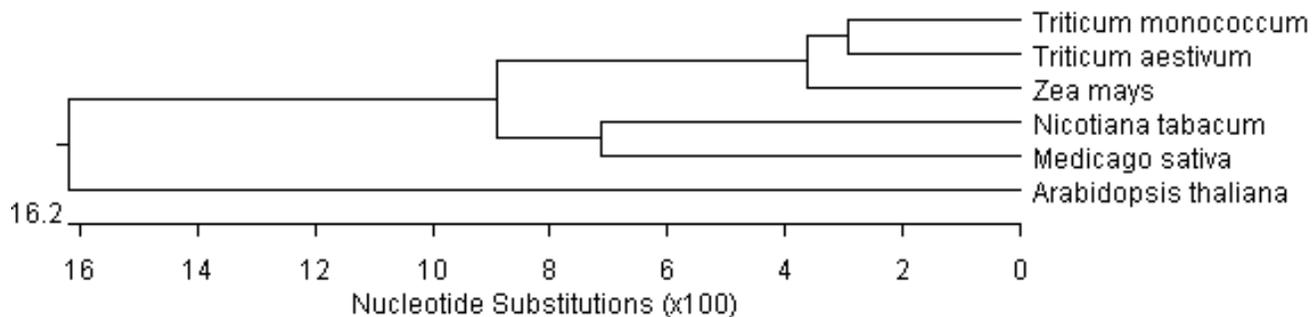
Expression analysis by quantitative real-time RT-PCR

The expression of mRNA for *TmGSK1* was examined by quantitative real-time RT-PCR under several different abiotic stresses on the Rotor-Gene 3000 system (Corbett Robotics). RT-PCR was performed using the all-in-one qPCR kit (Genecopoeia), following the manufacturers protocol. Primers were designed by primer express 2.0 software and synthesized by Beijing Sunbiotech Co., Ltd. A 189 bp product for *TmGSK1* cDNA was amplified using the following primers: T-forward 5' GTTTGGTCTGCTGGCTGTGTTCTT 3' and T-reverse 5' GTGCCATGGGTGAGCTTTGATTT 3'. A 118 bp product of einkorn wheat housekeeping gene β -actin was amplified as an internal control using the following primers: ACT-f 5' TGGCACCCGAGGAGCACCTG 3' and ACT-r 5' GCGACGTAC ATGGCAGGAACA 3'. The thermal cycling profile consisted of initial denaturation at 95°C for 3 min and 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. The resultant amplification was purified and sequenced, and showed 100% homology to target gene. To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. The specificity of PCR amplification was determined by constructing a melting curve after the polymerase chain reaction amplification, and negative controls containing RNase-free water instead of sample was run to confirm that the samples were not cross contaminated. Quantitation of relative expression was determined by the 2^{- Δ (Δ CT)} method (Livak and Schmittgen, 2001). Each sample was run in triplicate.

RESULTS AND DISCUSSION

cDNA clone and sequence analysis of the *TmGSK1* protein

The *TmGSK1* cDNA contained 1,543 bp with an open



Supplemental material 2. The phylogenetic tree of predicted protein sequence of GSKs from *T. monococcum* L., *T. aestivum*, *Z. mays*, *N. tabacum*, *M. sativa* and *A. thaliana*.

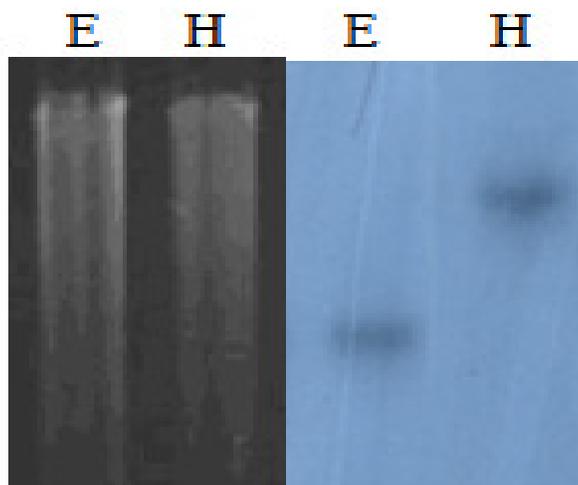


Figure 1. Southern-blot analysis of *TmGSK1*. Left: Agrose gel electrophoresis of the genomic DNA which was completely digested with EcoR I (E) and Hind III (H); Right: hybridization using the full-length sequence of *AbGSK1* as probe.

tol when it was stressed 6 h with NaCl and reached its maximum within 12 h after treatment; under wounding treatment, the expression pattern of *TmGSK1* was induced earlier than that under NaCl treatment (Figure 2b). Injury inducing its expression increased rapidly, up to 2-folds when it was injured just one hour. For abscissic acid (ABA) treatment, *TmGSK1* transcription was induced similar to that under wounding. Its expression increased 3-folds after treated with ABA for 1 h (Figure 2c). For drought treatment, it was 2.0 and 2.6 folds increase after treated with PEG6000 at 6 and 12 h (Figure 2d); low temperature (4°C) can induce its high expression too (Figure 2e). Expression pattern analysis indicates the possible involvement of *TmGSK1* in salt, wounding, ABA, drought and cold stress signal transduction pathways in einkorn wheat.

To understand a mechanism of abiotic stress signal transduction in plant, it is important to identify the components involved in the pathway. In this study, we

isolated the glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases from *T. monococcum* L. Previously, it has been shown that GSK3 are involved in a variety of biological processes. Present evidence indicates that plant GSKs are also involved in different processes such as hormone signaling, NaCl stress and other abiotic stresses. In accordance with these previous observations, we found in the present study that *TmGSK1* was also involved in the response of abiotic stresses such as salt stress, mechanical injury, abscissic acid hormone, cold and drought stress. Interestingly, as shown in Figure 2, the plants accumulate more *TmGSK1* mRNA response to all of these stresses. These means such stresses are inter-connected and may induce similar cellular signal transduction pathways. Moreover, these signal pathways may cross-talk on *TmGSK1* or *TmGSK1* may integrate all of these signal pathways in einkorn wheat. Stress signal activate the glycogen synthase kinase through different ways and substrates are phosphorylated and activity is

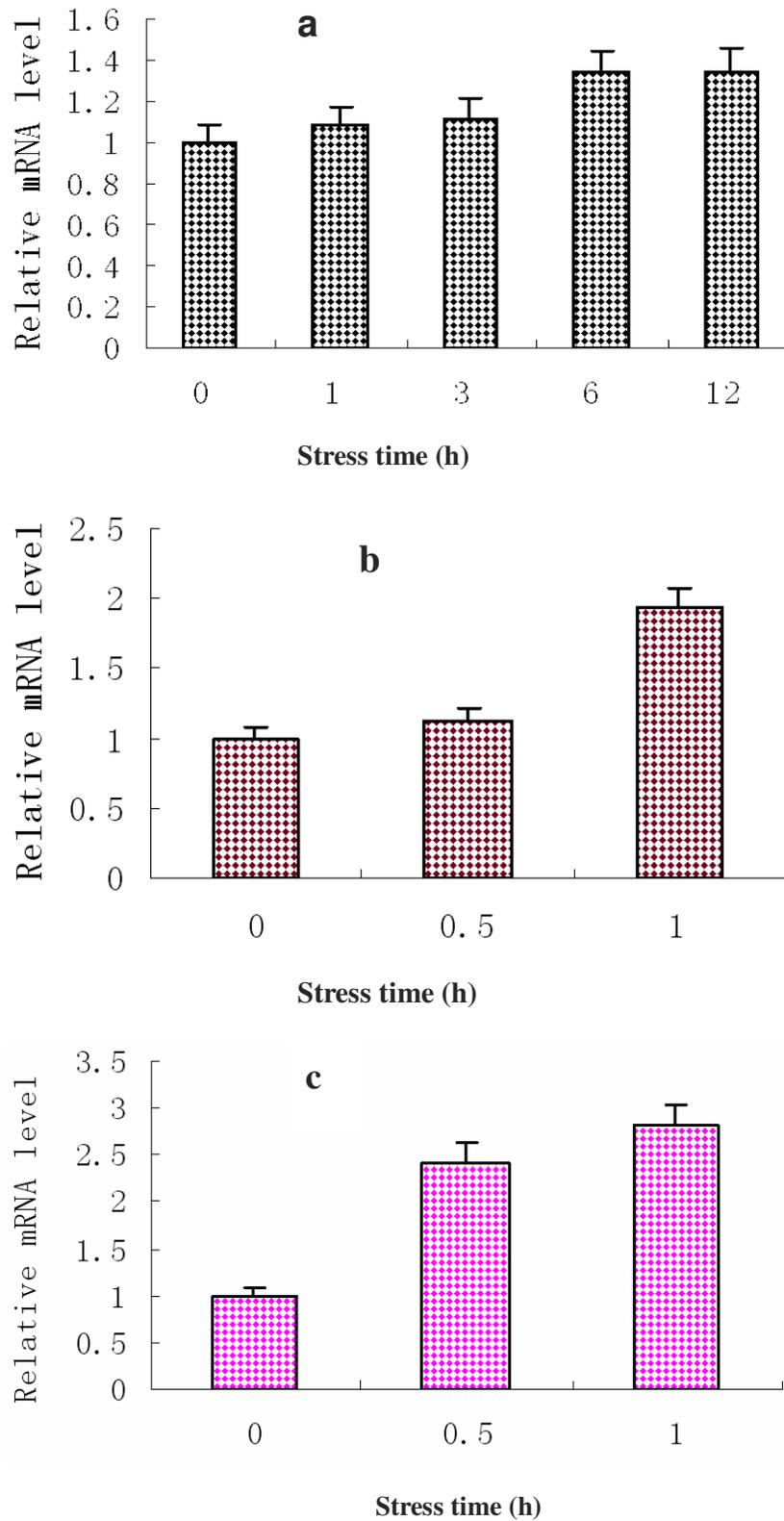


Figure 2. The relative mRNA level of *TmGSK1* under salt stress treatment (a); wounding treatment (b); ABA treatment (c); drought treatment (d); cold treatment (e). The quantitative real-time RT-PCR analysis of *TmGSK1* gene expression in *T. monocoecum* L in response to salt stress (1% NaCl), mechanical injury, ABA hormone, drought and cold treatment (4°C) using constitutively expressed β -actin gene as an internal control.

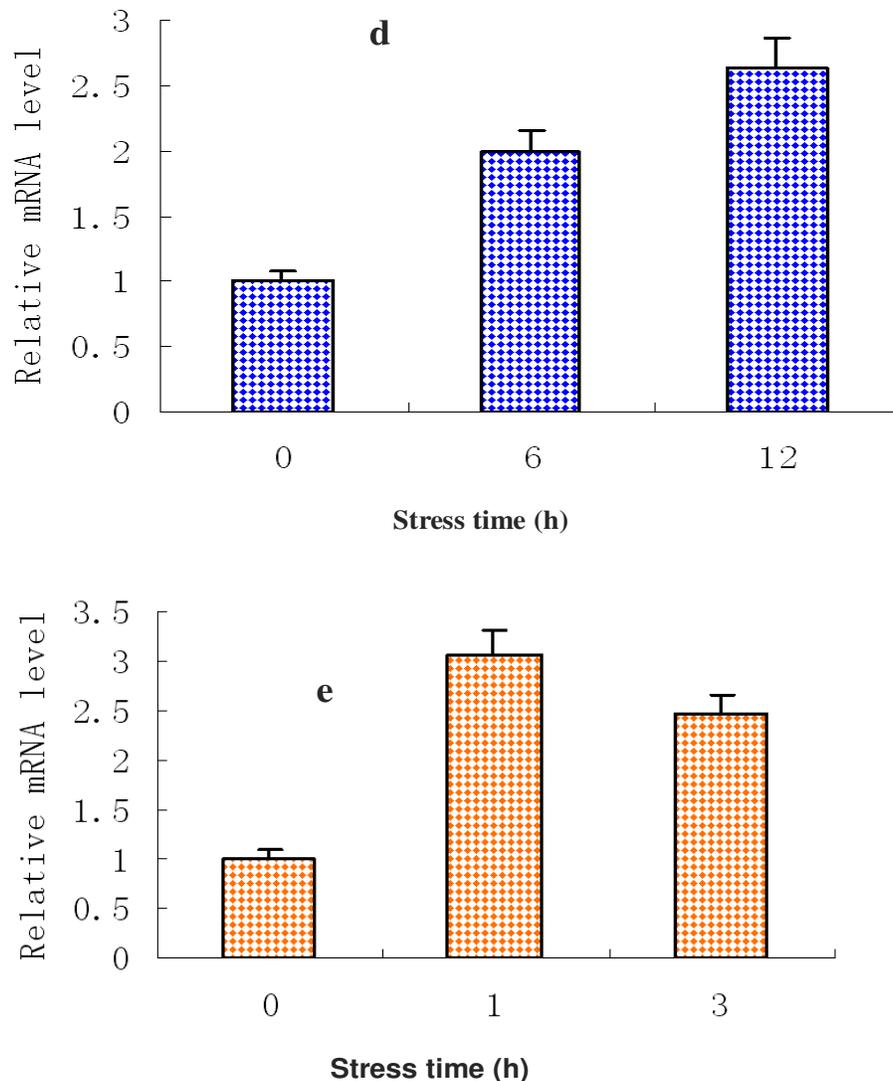


Figure 2. Contd.

regulated. Then, stress responsive genes express and start-up stress responsive mechanisms to re-establish cellular homeostasis and protect and repair damaged proteins and membranes. Therefore, TmGSK1 was shown to be a positive regulator commonly involved in the tolerance to salt, mechanical injury, ABA hormone, cold and drought in einkorn wheat. However, most mechanisms in this model are still unknown, and further investigation should be carried out to indicate the wheat plant abiotic stress responsive signal transduction in details.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (NO.31000165, 31171182), the Key Project of Chinese Ministry of Education (NO.209076) and

Foundation of science and technology research projects of Henan Province in China (No. 092300410099, 102300413213).

REFERENCES

- Almansouri M, Kinet JM, Lutts S (2001). Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant Soil*. 231(2): 243-254.
- Bajguz A, Hayat S (2009). Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiol. Biochem.* 47(1): 1-8.
- Bianchi MW, Guivarc'h D, Thomas M, Woodgett JR, Kreis M (1994). Arabidopsis homologs of the shaggy and GSK-3 protein kinases: molecular cloning and functional expression in escherichia coli. *Mol. Gen. Genet.* 242(3): 337-345.
- Chen GP, Ma WS, Huang ZJ, Xu T, Xue YB, Shen YZ (2003). Isolation and characterization of TaGSK1 involved in wheat salt tolerance. *Plant Sci.* 165(6): 1369-1375.
- Decroocq-Ferrant V, Van Went J, Bianchi MW, de Vries SC, Kreis M

- (1995). *Petunia hybrida* homologues of shaggy/zeste-white 3 expressed in female and male reproductive organs. *Plant J.* 67(6): 897-911.
- Dornelas MC, Wittich P, von Recklinghausen I, van Lammeren A, Kreis M (1999). Characterization of three novel members of the Arabidopsis SHAGGY-related protein kinase (ASK) multigene family. *Plant Mol. Biol.* 39(1): 137-147.
- Dornelas MC, Van Lammeren AA, Kreis M (2000). Arabidopsis thaliana SHAGGY-related protein kinases (AtSK11 and AtSK12) function in perianth and gynoecium development. *Plant J.* 21(5): 419-429.
- Einzenberger E, Eller N, Heberle-Bors E, Oscar V (1995). Isolation and expression during pollen development of a tobacco cDNA clone encoding a protein kinase homologous to shaggy/glycogen synthase kinase-3. *Biochimica et Biophysica Acta.* 31260(3): 315-319.
- Gill S, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, *Plant Physiol. Biochem.* 48(12): 909-930.
- Jonak C, Beisteiner D, Beyerly J, Hirt H (2000). Wound-induced expression and activation of WIG, a novel glycogen synthase kinase 3. *Plant Cell*, 12(8): 1467-1475.
- Jonak C, Heberle-bors E, Hirt H (1995). Inflorescence-specific expression of ATK-1, a novel arabidopsis thaliana homologue of shaggy/glycogen synthase kinase-3. *Plant Mol. Biol.* 27(1): 217-221.
- Katerji N, Van Hoorn JW, Hamdy A, Mastrorilli M (2004). Comparison of corn yield response to plant water stress caused by salinity and by drought, *Agric. Water Manage.* 65(2): 95-101.
- Knight H, Knight MR (2001). Abiotic stress signaling pathways: specificity and cross-talk. *Trends in Plant Science*, 6(6): 262-267.
- Koh S, Lee SC, Kim MK, Koh JH, Lee S, An G, Choe S, Kim SR (2007). T-DNA tagged knockout mutation of rice OsGSK1, an orthologue of Arabidopsis BIN2, with enhanced tolerance to various abiotic stresses. *Plant Mol. Biol.* 65(4): 453-466.
- Li J, Nam K (2002). Regulation of Brassinosteroid Signaling by a GSK/SHAGGY-Like Kinase. *Science*, 295(5558): 1299-1301.
- Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods*, 25(4): 402-408.
- Pay A, Jonak C, Bögre L, Meskiene I, Mairinger T, Szalay A, Heberle-Bors E, Hirt H (1993). The MsK family of alfalfa protein kinase genes encodes homologues of shaggy/glycogen synthase kinase-3 and shows differential expression patterns in plant organs and development. *Plant J.* 3(6): 847-856.
- Peng P, Zhao J and, Zhu Y (2010). Asami T., and Li J. A direct docking mechanism for a plant GSK3-like kinase to phosphorylate its substrates. *J. Biological Biol. Chem.* 285(32): 24646-24653.
- Piao HL, Lim JH, Kim SJ, Cheong GW, Hwang I (2001). Constitutive over-expression of AtGSK1 induces NaCl stress responses in the absence of NaCl stress and results in enhanced NaCl tolerance in Arabidopsis. *Plant J.* 27(4): 305-314.
- Piao HL, Pih KT, Lim JH, Kang SG, Jin JB, Kim SH, Hwang I (1999). An Arabidopsis GSK3/shaggy-Like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and Abscisic Acid. *Plant Physiol.* 119(4): 1527-1534.
- Rozhon W, Mayerhofer J, Petutschnig E, Fujioka S, Jonak C (2010). ASKtheta, a group-III Arabidopsis GSK3, functions in the brassinosteroid signalling pathway. *Plant J.* 62(2): 215-223.
- Sambrook J, Russell DW (2001). *Molecular cloning: A laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.
- Tavares R, Vidal J, van Lammeren A, Kreis M (2002). AtSK θ , a plant homologue of SG/GSK-3 marks developing tissues in Arabidopsis thaliana. *Plant Mol. Biol.* 50(2): 261-271.
- Wang wxWX, Vinocur B, Altman A (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*, 218(1): 1-14.
- Zhu JK (2001). Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* 4(5): 401-406.
- Zhu JK (2002). Salt and drought stress signal transduction in plant. *Annu. Rev. Plant Biol.* 53: 247-273.
- Zou HW, Wu ZY, Zhang XH, Wang YQ, Yang Q, Cao MQ, Huang CL (2008). Cloning and characterization of maize ZmASK1, a homologue to shaggy/GSK-3-Like gene, Involved in plant responses to abiotic stresses, *Acta Agron. Sinica*, 34(2): 184-191.