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

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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*, *Nicotiana tabacum*, *Medicago sativa* and *Arabidopsis thaliana*; therefore, the gene was named *TmGSK1* (*Triticum monococcum* Glycogen Synthase Kinase 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

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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 *alalfa* (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), abiotic stresses by real time quantitative polymerase chain reaction (RT-PCR). The RT-PCR was performed using the following primers for *TmGSK1*: T1-forward (5'- GTGTTGCTGTCGCTCTT-3') and T1-reverse (5'- TGCGGCGCTGATCGATCA-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-Qa 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).

**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 irrigated with distilled water. Two-week-old plants were treated with distilled water (as a control for Q-PCR analysis), high concentration of salt (170 mM NaCl), mechanical injury (shearing), ABA hormone (100 µM), 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 DNasel (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'- GTGTTGCTGTCGCTCTT-3') and T1-reverse (5'- TGCGGCGCTGATCGATCA-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-Qa 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-week-old einkorn wheats 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 µg 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×SSC and hybridized with the probe, which was labeled with \([a-\text{32P}]\text{CTP}\) by Random Primer DNA Labeling kit(Takara), for 16 h at 65°C. The blots were washed once in 0.1% SDS, 2×SSC for 15 min at 65°C and twice in 0.1% SDS, 0.1×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'- GTTGGTCTGTCGCTCTT-3' and T-reverse 5'- GTGCCATGGGTAGCTTTGATT-3'. A 188 bp product of einkorn wheat housekeeping gene β-actin was amplified as an internal control using the following primers: ACT-f 5' TGGCACCCGAGGAGCACCCTG 3' and ACT-r 5' GCGACGTAC ATGGCAGAACC 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 etidium 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^(-ΔΔCT) 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 1. Alignment of sequence of GSKs predicted protein from T. monococcum L., T. aestivum, Z. mays, N. tabacum, M. sativa and A. thaliana. The black shading indicates identical amino acids. Amino acid sequences were aligned with the MegAlign program (DNAstar software (DNASTAR, Inc., Madison, WI, USA)) using the CLUSTALW method.

Southern analysis of TmGSK1

Southern analysis of genomic DNA digested separately with two different restriction enzymes, EcoRI and HindIII, was performed to estimate the copy number of TmGSK1 in the einkorn wheat genome. As shown in Figure 1, the banding pattern was produced with the TmGSK1 full-length probe (1,543 bp) and a high-stringency wash. Only one hybridizing band in each digestion was observed, indicating that the TmGSK1 sequence was present as a single copy in the einkorn wheat genome.

Quantitative real-time RT-PCR of the TmGSK1 gene in response to various abiotic stresses

The quantitative real-time RT-PCR analyses of TmGSK1 during various abiotic stresses are shown in Figure 2. Constitutively expressed β-actin gene was used as an internal control. TmGSK1 expression was upregulated in these abiotic stresses. Accumulation of TmGSK1 mRNA increased step by step under salt stress (Figure 2a). Its expression was up to 1.5-folds when compared to the control.

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.

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 interconnected 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. monococcum L. in response to salt stress (1% NaCl), mechanical injury, ABA hormone, drought and cold treatment (4°C) using constitutively expressed \(\beta\)-actin gene as an internal control.
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.

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