Special article

Systematic analysis of glycogen synthase kinase 3 genes in rice reveals their differential responses to phytohormones and abiotic stresses

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Abstract The glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases are serine/threonine protein kinases involved in a variety of biological processes. In this study, nine *GSK*3-like genes (*OsGSK*1-9) were identified in rice, and they are distributed on six chromosomes, and the distribution pattern is related to the chromosomal block duplication events in rice. The OsGSK proteins can be classified into four subgroups. The expression patterns of *OsGSK* genes were investigated in various tissues and organs of rice and in the seedlings treated with phytohormones and abiotic stresses. The results suggest that most of *OsGSK* genes have high expression level in the whole life cycle, and they are responsive not only to different phytohormones (such as abscisic acid, auxin, and brassinosteroid) but also to drought and salt stresses, implying that *OsGSK* genes may have important roles in development and stress responses in rice.

Key words *GSK*; phytohormone; abiotic stress; expression profile; protein kinase

CLCN S 511.503.53 **Document code** A **Article number** 1000-2421(2012)01-0001-09

The glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases are highly conserved serine/threonine protein kinases that are involved in a variety of cellular processes including cell proliferation, cell differentiation, cytoskeleton dynamics, and programmed cell death, and they are regulated by phosphorylation or protein-protein interaction in animal cells[1]. GSK3 was originally identified in mammals as a cytoplasmic serine/threnonine protein kinase that regulates metabolism of storage carbohydrate glycogen[2]. GSK3 homologues have been found in all eukaryotes. Plants possess a large gene family of *GSK*3/*SHAGGY*-like kinases (*GSKs*). Analysis of the *Arabidopsis* genome revealed an existence of 10 *GSK* genes[3]. Genetic and biochemical studies have revealed that different plant *GSKs* are involved in diverse processes, including hormone signaling, development and stress responses. *AtGSK*1 can complement the salt-sensitive phenotype of yeast calcineurin mutants[4], and it is induced by salt stress and abscisic acid treatment. Overexpression of *AtGSK*1 resulted in enhanced salt and drought tolerance[5]. In *Medicago sativa*, a *GSK*3 gene (*WIG*) is induced by wounding. Although *WIG* transcript is hardly detectable in mature leaves, and *WIG* mRNA accumulates rapidly after wounding[6]. AtSK11 and AtSK12, two *SHAGGY*-like kinase from *Arabidopsis*, seem to regulate floral meristem patterning since reducing the transcript levels of these two genes by antisense suppression approach led to an increase in the number of perianth organs and an alteration of the apical-basal patterning of gynoecium[7]. *Arabidopsis SHAGGY*-like kinase (also termed ASK)

Received date: 2011-04-28

Grants: The National Natural Science Foundation of China (30725021, 30830071, and 30921091) and the National Special Key Project of China on Functional Genomics of Major Plants and Animals (2006AA10A103)

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genes have diverse tissue or organ-specific expression patterns. *ASKζ* is expressed in the whole embryo during its development while the expression of *ASKη* is limited to the suspensor cells, but no signal was detected for *ASK*11, *ASK*12 and *AtGSK*1 in developing embryos[8]. Cloning of the *BIN*2 (brassinosteroid-insensitive 2) locus, also named as *ASKη* and identical to *UCU*1 and *DWF*12, revealed that BIN2 participates in brassinosteroid signaling and acts as a negative regulator to control the brassinosteroid signaling[9].

The phylogenetic diversification of glycogen synthase kinase3/*SHAGGY*-like kinase genes in the whole plant kingdom have also been reported[3]. Rice is a model plant of monocot species for functional genomics and gene function studies; however, very limited knowledge of the *GSK* gene family is available in this species. So far, only one GSK protein, OsGSK1, has been reported in rice and this protein may functions in abiotic stress signaling and was proposed as an ortholog of BIN2 in rice[10]. In this study, an attempt was made to find out all *GSK* genes existed in rice genome through a systematic analysis. Sequence data mining resulted in the identifications of nine *GSK* genes in rice (OsGSK1-9). We further investigated the gene expression patterns of these genes under different hormone and abiotic stress treatments. Meanwhile, the expression profiles of *OsGSK* genes in the entire life cycle of rice were checked. In addition, informative discussion was made on the stress responses and BR-auxin signaling crosstalk based on the expression profiles of the family and the possible OsGSK-interacting proteins.

1 Materials and methods

1.1 Identification and sequence analysis of OsGSK family

The amino acid sequences of AtGSK in *Arabidopsis* were downloaded from The *Arabidopsis* Information Resource (TAIR: http://www.arabidopsis.org). For identification of GSK homologs in rice, the Knowledge-based Oryza Molecular Biological Encyclopedia (KOME, http://www.cdna01.dna.affrc.go.jp/cDNA), the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST), and The Institute for Genomic Research (TIGR) database (http://www.tigrblast.tigr.org/euk-blast) resources were used. The PFAM database (http://www.sanger.ac.uk/Software/Pfam/) was used to check if the browsed protein sequence has a highly conserved kinase domain as a GSK protein family member.

Each of the *OsGSK* genes was positioned on rice chromosome pseudomolecules available at TIGR (release 6) by the BLASTN search. The distinctive name for each of the *OsGSK* identified in this study is given according to its position from the top to the bottom on the rice chromosomes 1 to 12. The presence of *OsGSK* genes on duplicated chromosomal segments was investigated by checking the segmental genome duplication of rice available at TIGR (http://rice.plantbiology.msu.edu/segmental_dup/500kb/segdup_500kb.shtml) with the maximum length of 500 kb permitted for collinear gene pairs.

The phylogenetic tree was constructed using PHYLIP software. A series of programs including Sequence Boot, Protdist, Maximum-likelihood and consensus were used to obtain boot strap values. MEGA 3.0 software was used to make a readable form of phylogenetic tree by conducting 1 000 replications.

Information about the number of amino acids, molecular weight, theoretical isoelectric point (pI), and amino acid composition were obtained by EXPASY PROTOPARAM tool (http://www.expasy.org/tools/protparam.html). The interacting proteins of OsGSK genes were obtained from Rice Kinase Database (RKD: http://rkd.ucdavis.edu/).

1.2 Microarray data resource for expression profiling analysis

Expression profiles of *OsGSK* gene family in an elite hybrid rice parent Minghui 63, was extracted from CREP database (http://crep.ncpgr.cn).

Hierarchical cluster analysis of the expression patterns of *OsGSK* family was based on $\log_{10}$-transformed signal values of the genes in 30 representative organs/tissues.

1.3 Plant growth and phytohormone and stress treatments

The germinated seeds of indica rice Minghui 63 were placed on wet filter papers in Petri dishes and incubated in an incubator of 28 ℃ and 14 h/10 h (L/D) photoperiod for 3 weeks, and then subjected to treatments. Phytohormone, drought and salt treatments were conducted as described in previous study[11].

1.4 Quantification of gene expression

RT-PCR was carried out by using the RNA extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA).) according to the manufacturer's instructions. All gene-specific primers were designed based on the cDNA sequences using the primer 3 software for RT-PCR. The specific primer for the rice *Actin* gene (*X*15865) was served as an internal control. Reactions were performed with r*Taq* polymerase (Takara Biotechnology, Dalian, China) on Gene AMP PCR system 9700 (Applied Biosystem, USA), with the following profile: 4 min at 94 ℃ for pre-denaturation, followed by 29 cycles of 45 s at 94 ℃, 40 s at 57 ℃, and 40 s at 72 ℃, and a final 5 min extension at 72 ℃. Each PCR was repeated for three times. The quantitative real-time PCR analysis was performed as described before[11].

Each pair of real-time primers designed by using Primer Express Software (Foster City, CA, USA) was checked by the BLAST program in rice genomic sequence available in TIGR database to ensure that the primers amplify a unique and desired cDNA segment. The primers for real-time PCR are listed in Table 1.

Table 1　RT-PCR primers used in this study

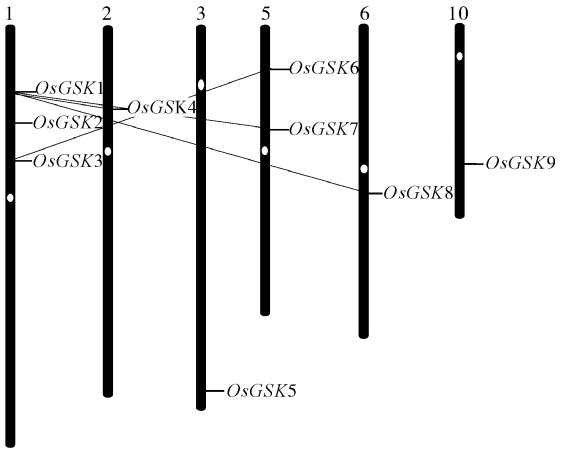
Gene	Forward primer (5′ - 3′)	Reverse primer(5′ - 3′)
*OsGSK*1	GATTTTCCACAAGAGAATGC	CTCTGGTACTGAGTCCTTGC
*OsGSK*2	TGAGTATGTGCCTGAAACTG	ATTTCCTCCCTTGTAGGTGT
*OsGSK*3	GAACTACGAGATCCAAATGC	CTGATAGAGGCTCTGGAATG
*OsGSK*4	ACCGAGTACACGACATCAAT	ATGCTCTGGTATCAACCTGT
*OsGSK*5	CGAGCACTTGCTTACATACA	CTTACAAGGTCCACTGCTTC
*OsGSK*6	AGTATGTGCCAGAGACTGCT	GCACTTAATCTCCTCTCGTG
*OsGSK*7	AGCTACCCACAACTTCTTCA	GTTATTCATCAGAGGCATGG
*OsGSK*8	GAGTTACGAGAACCACATGC	GGAACATACAGCAAAGGAAG
*OsGSK*9	CTGGAGAAAGTGGTGTTGAT	ACACCTATCCGCTGTTCTAA
Osactin	CTCAACCCCAAGGCTAACAG	ACCTCAGGGCATCGGAAC

2 Results and analysis

2.1 Identification of rice *GSK* genes

Yoo et al[3] collected GSK homologues from representative plants and constructed a comprehensive phylogenetic tree, which provided a general view of the structure of these genes in plant kingdom. To obtain more detail information of the GSK family specifically for rice, we re-searched the predicted proteins of rice genome in the TIGR database by using the conserved kinased domain of GSK proteins as a query, and nine putative GSK homologues were identified. All the protein and cDNA sequences for were then downloaded from TIGR and KOME databases. The Pfam database was used to confirm the conserved domain for the GSK family in rice. Results suggest that the rice genome contains nine GSK homologues and all of them have the complete kinase domain. The chromosomal locations of the *OsGSK* genes were identified based on BLASTN search against the rice chromosome pseudomolecules available at TIGR. To uniform the nomencalture of GSK homologues in rice, we named nine *OsGSK* genes from *OsGSK*1 to *OsGSK*9 according to the order of their locations in the rice chromosomes. The nine *OsGSK* genes

are distributed on six of the 12 rice chromosomes: three on chromosome 1; one each on chromosome 2,3,6,and 10; and two on chromosome 5 (Fig. 1). By checking the TIGR rice segmental duplication with the maximum length of 500 kb for collinear gene pairs, we noticed that *OsGSK*4, *OsGSK*7 and *OsGSK*8 may be duplicated from *OsGSK*1; and *OsGSK*3 and *OsGSK*6 are located in the corresponding duplicated regions in chromosome 1 and 5, respectively (Fig. 1). However, no tandem duplication was found in the *OsGSK* family. These results suggest that genomic duplication may contribute to the amplification of *OsGSK* family in rice.



White ovals on the chromosomes indicate the positions of centromeres. The chromosome numbers are indicated at the top of each bar.

Fig. 1 Genomic distribution and duplication patterns of *OsGSK* genes on rice chromosomes

EXPASY analysis indicated that the OsGSK protein sequences have similar isoelectric point (pI) values (ranging from 7.60 in OsGSK9 to 8.89 in OsGSK2). The sizes of OsGSKs vary from 402 (OsGSK4) to 470 (OsGSK9) amino acids and the molecular weights of these deduced OsGSK proteins range from 45.2 ku (OsGSK1) to 52.6 ku (OsGSK9) (Table 2). The structures of *GSK* genes in *Arabidopsis* and rice are highly conserved, and most of them have 12 exons interrupted by 11 introns.

The OsGSK proteins appear to be highly conserved in the kinase domain. In contrast, the N and C-terminal regions are highly variable. It is interesting to note that OsGSK9 has a short extra N-terminal sequence (Fig. 2), which may imply that the function of OsGSK9 may be different from other OsGSKs.

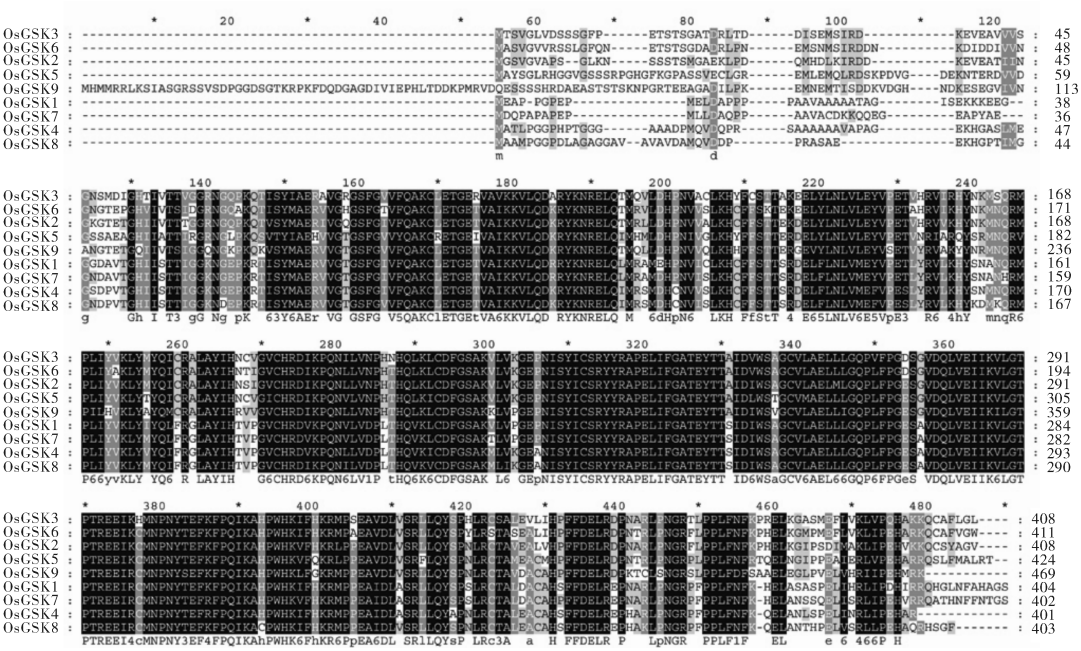
Sincethe functionally characterized GSK proteins are mainly from *Arabidopsis*, we re-examined the phylogenetic relationship of rice and *Arabidopsis* GSK proteins. An unrooted tree was constructed based on the alignment of full-length GSK protein sequences and the result showed that *Arabidopsis* and rice GSKs can be classified into four subgroups (Fig. 3), which is consistent with the phylogenetic result based on exon and intron structures reported previously[3].

Table 2 Generic information of GSK family in rice

Name	cDNA accession No[1)]	Locus ID[2)]	ORF length/bp	Protein length (AA)	MW/ku	Isoelectric point
*OsGSK*1	AK099863	LOC_Os01g10840	1 215	405	45.2	8.12
*OsGSK*2	AK099742	LOC_Os01g14860	1 227	409	46.1	8.89
*OsGSK*3	AK120194	LOC_Os01g19150	1 314	438	49.5	8.79
*OsGSK*4	AK073725	LOC_Os02g14130	1 206	402	44.8	8.40
*OsGSK*5	AK070062	LOC_Os03g62500	1 275	425	48.2	8.58
*OsGSK*6	AK058276	LOC_Os05g04340	1 236	412	46.8	8.46
*OsGSK*7	AK102147	LOC_Os05g11730	1 209	403	45.5	7.95
*OsGSK*8	AK100950	LOC_Os06g35530	1 212	404	45.3	8.07
*OsGSK*9	AK072390	LOC_Os10g37740	1 410	470	52.6	7.60

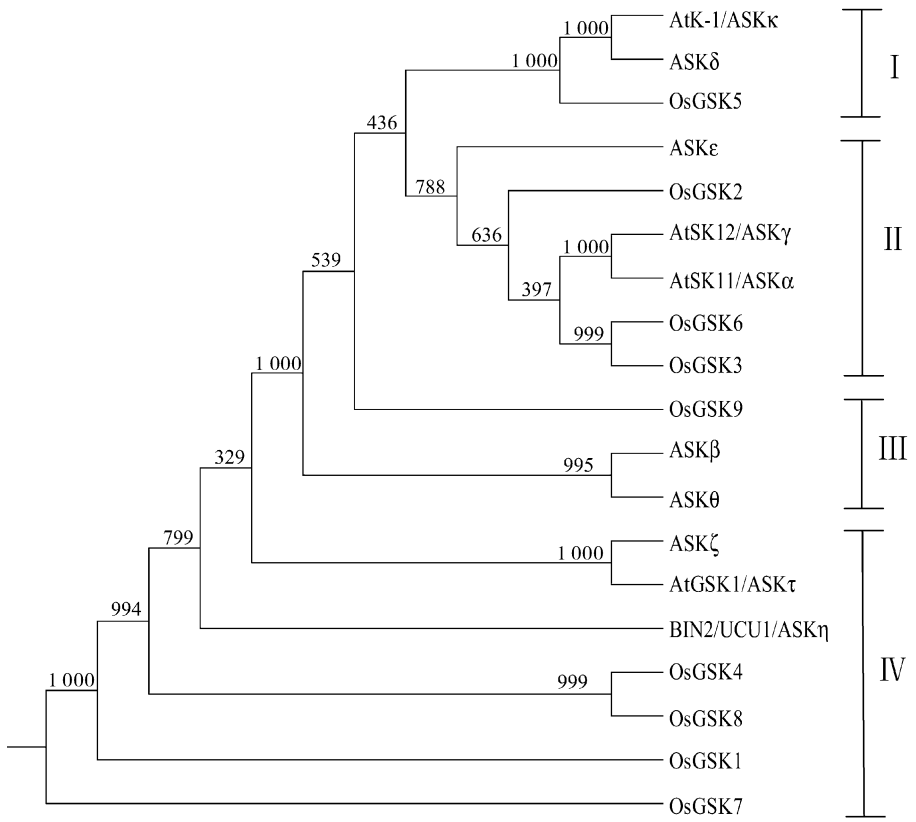
1) Accession numbers of full-length cDNA sequence available at KOME (http://cdna01.dna.affrc.go.jp/cDNA/);

2) Locus ID of each *OsGSK* gene on rice chromosome pseudomolecules available at TIGR (release 6).



The respective amino acid position is given on the top of each sequence and the protein names were indicated at the left side of the figure. The region with dark background corresponds to kinase domain.

Fig. 2　Sequence alignment of rice GSK proteins by Clustal X program



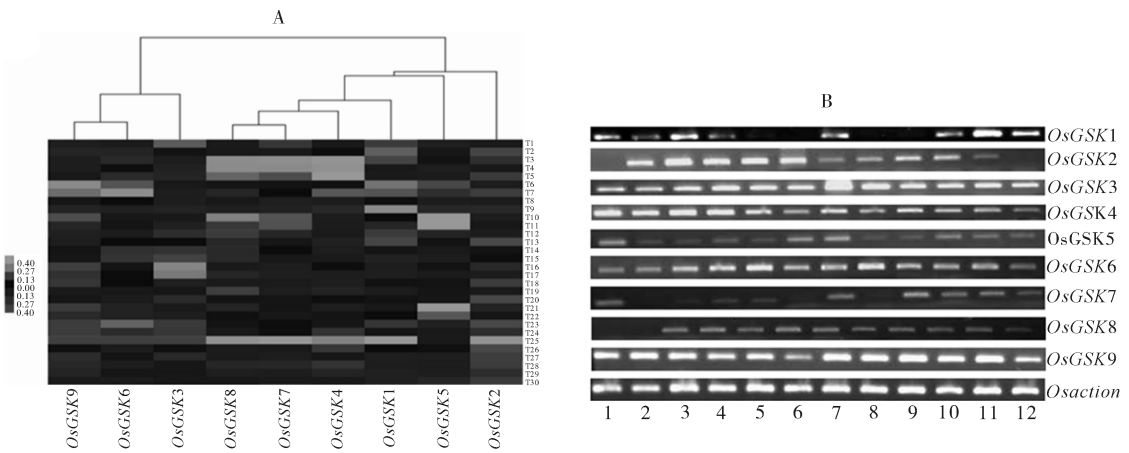
An unrooted tree was generated using MEGA 3.0 program by maximum-likelihood method.

Fig. 3　Phylogenetic trees of *Arabidopsis* and rice GSK proteins

2.2 Expression profiles of OsGSK genes in the whole life cycle of rice

Expression profiles of *OsGSK* genes were prechecked by extracting the microarray expression data from CREP database (http://crep.ncpgr.cn). A total of 30 representative tissues were selected for expression level comparison of the genes in *OsGSK* family. Most of the *OsGSK* genes had high levels in all the organs/tissues, suggesting that these *OsGSK* genes may be involved in a wide spectrum of stages during the growth and development of rice. From the microarray data, two genes (*OsGSK*2 and *OsGSK*5) had relatively high expression in panicle; three genes (*OsGSK*3, *OsGSK*6 and *OsGSK*9) were predominantly expressed in stamen; four genes (*OsGSK*1, *OsGSK*4, *OsGSK*7 and *OsGSK*8) were constitutively expressed (Fig. 4-A).

RT-PCR was performed to confirm the expression profiles in some selected tissues (callus, seedling, leaf, flag leaf, sheath, stem, root, stamen, and panicle from different stages). The RT-PCR results are generally in agreement with the microarray data (Fig. 4-B). The differential expression profiles of *OsGSK* genes suggest that different members of the family may have both overlapping and distinct functions at different developmental stages.



A: Hierarchical analysis of expression profiles of *OsGSK* family in 30 organs/tissues. The signal value is $\log_{10}$-transformed and subjected to a complete linkage hierarchical clustering analysis with treeview program. The 30 organs/tissues are the same as used in our previous report[11]. B: Expression levels of the *OsGSK* genes detected by RT-PCR analysis. 1: Callus; 2: Seedling; 3: Leaf at tillering stage; 4: Flag leaf; 5: Sheath; 6: Stem; 7: Root at tillering stage; 8: Stamen; 9-12: Panicle at different stages (from young to old).

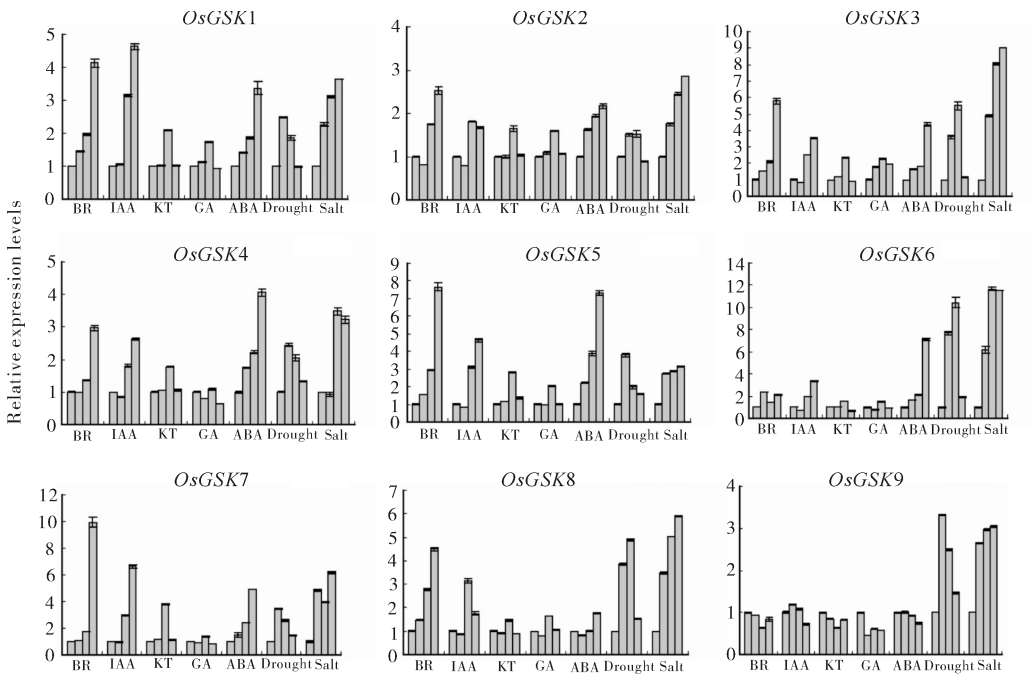
Fig. 4 Expression profiles of *OsGSK* family in tissues and organs

2.3 Response of rice GSK genes to different phytohormone treatments

BIN2, one of the *GSK* members in *Arabidopsis*, negatively regulates brassinosteroid signaling pathway and can phosphorylate ARF2 to regulate auxin signaling pathway[12]. To check if the *OsGSK* genes are also involved in response to different phytohormones in rice, real-time PCR was carried out to quantify the expression levels of *OsGSK* genes under treatments of different phytohormones (Fig. 5). Under the BR (24-epiBL used) treatment, five genes (*OsGSK*1, *OsGSK*3, *OsGSK*5, *OsGSK*7 and *OsGSK*8) were strongly induced (>4-fold), two genes (*OsGSK*2 and *OsGSK*4) were slightly (about 3-fold) induced, while two genes (*OsGSK*6 and *OsGSK*9) had no response to the treatment. Under the IAA treatment, four genes (*OsGSK*1, *OsGSK*3, *OsGSK*5 and *OsGSK*7) were significantly induced, four genes (*OsGSK*2, *OsGSK*4, *OsGSK*6 and *OsGSK*8) were slightly induced, but the *OsGSK*9 showed no response. The induction kinetics of most genes by IAA was similar to that by 24-epiBL. In the KT treatment, six genes (*OsGSK*1, *OsGSK*2, *OsGSK*3, *OsGSK*4,

*OsGSK*5 and *OsGSK*7) were slightly induced, while the other three genes (*OsGSK*6, *OsGSK*8 and *OsGSK*9) were not induced.

In the GA treatment, no gene showed significant induction. Six genes (*OsGSK*1, *OsGSK*3, *OsGSK*4, *OsGSK*5, *OsGSK*6 and *OsGSK*7) were significantly induced (>3-fold) by ABA, *OsGSK*2 was slightly induced, while the other two genes (*OsGSK*8 and *OsGSK*9) showed no response to ABA.



X-axes are treatment/time course. Four columns in each group, from left to right, stand for time course 0, 3, 6, 12 h. *Y*-axes are scales of relative expression levels (The bars indicate standard errors).

Fig. 5　Real-time PCR analysis of the *OsGSK* genes under phytohormone and stress treatments

Taken together, except *OsGSK*9 that showed no response to any of the phytohormone treatments, all the other *OsGSK* genes were responsive to one or more phytohormones, and ABA, indicating that *OsGSK* genes may be involved in the cross-talks of signaling pathways involving these hormones.

2.4　Response of OsGSK genes to abiotic stresses

*OsGSK*1 has been reported with a positive role in abiotic stress tolerance[10]. To investigate whether other *OsGSK* genes are also involved in abiotic stresses, real-time PCR were carried out to check the expression level changes of this family under salt and drought treatments (Fig. 5). Result showed that all the *OsGSK* genes were responsive to drought treatment and the expression levels peaked at 3-6 h and then decreased to the basal expression level at 12 h after treatment. Among them, three genes (*OsGSK*3, *OsGSK*6, and *OsGSK*8) showed significant induction (>6-fold) by drought treatment. In the salt treatment, all the *OsGSK* genes were gradually induced through the time course. Among them, three genes (*OsGSK*3, *OsGSK*6, and *OsGSK*8) were strongly up-regulated (>6-fold) and the other *OsGSK* genes were slightly (about 3-fold) induced.

3　Discussion

To date, only a few plant GSK proteins have been functionally characterized for their roles in BR signaling, development and stress tolerance. It is known that BRs are related to a variety of abiotic stresses including high and low temperatures,

drought, and salinity and exogenous BR can increase stress tolerance[13]. BIN2 acts as a negative regulator in BR signaling pathway and belongs to *GSK* family. T-DNA insertion mutant of *OsGSK*1 showed significantly increased tolerance to cold, heat, salt, and drought stresses and overexpression of *OsGSK*1 exhibited stunted growth phenotype, implying that *OsGSK*1 may function as *BIN*2 orthologue and it is involved in the modulation of abiotic stress tolerance in rice[10]. A novel *Medicago sativa* GSK-3-like kinase Msk4 has been proposed as an important regulator for stress tolerance and carbohydrate metabolism[14]. In this study, the real-time PCR results showed that all the *OsGSK* genes are responsive to drought and salt treatments. By checking the microarray data of rice under other stress treatments, we noticed that most of the *OsGSK* genes are also responsive to cold, heat and oxidative stresses (data not shown). These results imply that *GSK* family may play diverse roles in modulating multiple abiotic stress responses in rice.

Recently, intensive attention has been received to the cross-talks between different phytohormone-mediated signaling pathways, especially for the field studying the direct molecular links between different hormone signaling pathways. Mouchel et al[15] reported that *BRX* acts at the nexus of a feedback loop that maintains a threshold BR level to permit optimal auxin action. Vert et al[12] found one of the molecular links between BR and auxin was the interaction between BIN2 and ARF2 and provided strong evidence to support that BIN2 regulates both DNA binding and transcriptional activity of ARF2. Our previous study showed that *OsIAA*1 is involved in both auxin and brassinosteroid hormone responses[16]. Recently, Zhang et al[17] provided strong evidence to demonstrate that ABA inhibits the primary signaling outputs of BR through ABI2 and ABI1, and BR and ABA signaling cross-talk may occur at the downstream of BR receptor complex. In this study, most *OsGSK* genes are responsive to BR, auxin and ABA, which further supports the diverse roles of GSK family in the cross talks of multiple hormone signaling pathways.

In the RKD database (http://rkd.ucdavis.edu/), potential interacting proteins for OsGSK2, OsGSK3, OsGSK4 and OsGSK5 were presented based on yeast two-hybrid screening results. It is very interesting to notice that OsGSK3 and OsGSK5 have the same two putative interacting proteins (LOC_Os02g46620 and LOC_Os06g43990), while OsGSK4 interacts with OsARF2. OsGSK4 shows high identity of amino acid sequence with BIN2 in *Arabidopsis*, suggesting OsGSK4 may have similar function as BIN2 in rice. Meanwhile, OsARF2 shows high similarity with *Arabidopsis* ARF2. OsARF2-overexpressed rice showed similar phenotypes as of ARF2-overexpression in *Arabidopsis* (Song and Xiong, unpublished data), suggesting that OsARF2 may have the same function as ARF2. Vert et al[12] showed that BRs can synergistically increase seedling sensitivity to auxin and proved that the combined treatment with both hormones can increase the magnitude and duration of gene expression. However, how these two hormones regulate the plant growth and responses to environmental stimuli through the precise functions of key regulators such as Aux/IAAs, ARFs, and GSKs by protein-protein interaction in molecular level is still a mystery. Therefore, the interaction of OsGSK4 with OsARF2, once confirmed, could be a good start point to investigate the cross-talk of BR and auxin signaling pathways in rice.

In summary, GSK family appears to have diverse roles in plants. In this study, we provide basic but valuable information for future studies on elucidating the precise roles of *OsGSK* genes in signaling, development, and stress responses. Further studies should be focused on how these family members are involved in development at different stages or specific stress response with special attention to signaling cross-talks of related hormones.

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水稻 GSK 基因家族的鉴定及其对多种激素和逆境应答的表达量分析

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摘要　通过序列比对分析鉴定出 9 个 *GSK* 同源基因（命名为 *OsGSK*1-9），它们分布在水稻的 6 条染色体上。聚类分析表明预测的 OsGSK 蛋白和其他植物中的 GSK 蛋白可被分为 4 个亚组。通过实时定量 PCR 进一步分析了 *OsGSK* 基因家族的基因在水稻各种组织和器官以及在多种逆境胁迫和植物激素处理条件下的表达量。结果表明：大多数 *OsGSK* 基因在水稻全生育期都有较高的表达量并且受多种激素（如脱落酸、生长素、油菜素内酯）和逆境（如干旱和盐胁迫）胁迫诱导表达，表明 *OsGSK* 基因家族在水稻发育和逆境适应过程中可能起重要作用。

关键词　*GSK*；植物激素；非生物逆境；表达谱；蛋白激酶

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