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ORIGINAL ARTICLE

Differential Gene Expression of Two Outward-Rectifying Shaker-Like Potassium Channels OsSKOR and OsGORK in Rice

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Abstract Shaker-like potassium channels are highly conserved voltage-dependent ion channels in plants. Rice, a monocot model plant, has similar numbers of shaker-like potassium channels as Arabidopsis. Although several inward-rectifying potassium channels have been identified, no outward-rectifying potassium channels have yet been reported for rice. Here, we identified two outward-rectifying shaker-like potassium channels in rice, with high amino acid sequence similarities to Arabidopsis stelar K^+ outward rectifier (SKOR) and guard cell outward rectifying K^+ channel (GORK). To characterize these channels we monitored their expression patterns in several tissues. qRT-PCR and promoter-GUS analysis showed that *OsSKOR* is expressed in root vascular tissues, flower, and seed scutellum. *OsGORK* was expressed to some degree in most tissues, such as leaf blade, node, leaf sheath and root, but at high levels in flowers. This is the first report characterizing rice outward-rectifying potassium channels and contributes to understanding the regulation of potassium homeostasis in monocot plants.

Key words: OsGORK, OsSKOR, Outward-rectifying potassium channel, Rice, Root, Shaker like potassium channel

Introduction

Potassium (K^+) is the most prevalent cation and constitutes approximately 10% of the dry weight of plants. K^+ plays roles in multiple processes, including electrical neutralization of anionic groups, control of membrane polarization, and regulation of osmotic pressure in the cell. Water potential and turgor pressure of plant cells is regulated mainly by K^+ uptake and release. Thus, K^+ uptake, transport, export and storage have to be regulated elaborately in response to environmental changes.

Over the past decades several different families of K^+ transporters have been identified, such as shaker-like, KCO, and KUP/HAK/KT transporters and HKTs in plants (Maser et al. 2001; Very and Sentenac 2003). Among them, shaker-like K^+ channels mainly regulate K^+ homeostasis in plants (Hedrich 2012). AtKAT1, of the shaker-like K^+ channel family, was the first channel for which the cDNA was cloned in plants (Anderson et al. 1992). The shaker-like gene family consists of nine members in Arabidopsis. The members are classified into three subgroups based on their current rectification properties in Arabidopsis: inward, weakly inward, or outward (Very and Sentenac 2003). KAT1, KAT2, AKT1 and SIPK are inward-rectifying K^+ channels. KAT1 regulates stomatal opening by uptake of K^+, which increases the osmotic pressure in guard cells (Very and Sentenac 2003). AKT1 is involved in high-affinity K^+ uptake through the root in Arabidopsis (Nieves-Cordones et al. 2014). SIPK is involved in K^+ uptake in pollen and pollen tube development (Mouline et al. 2002). Seven of nine shaker-like K^+ channels have characteristics of inward rectifiers and only two channels, stelar K^+ outward rectifier (SKOR) and guard cell outward rectifying K^+ channel (GORK) are known to be outward-rectifying K^+ channels (Very and Sentenac 2003). SKOR is expressed in root stelar tissues and loads K^+ into the xylem, whereas GORK is expressed in guard cells and induces stomatal closing through K^+ export from guard cells in Arabidopsis (Gaymard et al. 1998; Ache et al. 2000).

Although more than 30 genes from ~10 plant species have been identified for inward-rectifying K^+ channels, only 5 genes from 4 species are known for outward-rectifying K^+ channels. These comprise GORK and SKOR in *Arabidopsis thaliana*, PTORK (*Populous tremula* outward rectifying K^+ channel), SPORK (*Samanea saman* pulvinus outward rectifying

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K^+ channel) and ZORK (*Zea mays* outward rectifying K^+ channel) (Gaymard et al. 1998; Ache et al. 2000; Moshelion and Moran 2000; Langer et al. 2002; Buchsenschutz et al. 2005). To characterize outward-rectifying K^+ channels in rice, a model plant for monocots, we isolated two candidate outward-rectifying K^+ channels based on amino acid similarity and analyzed their tissue-specific expression patterns and subcellular localization. Our findings supported the classification of two outward-rectifying K^+ channels, *OsGORK* and *OsSKOR*, in rice.

Results

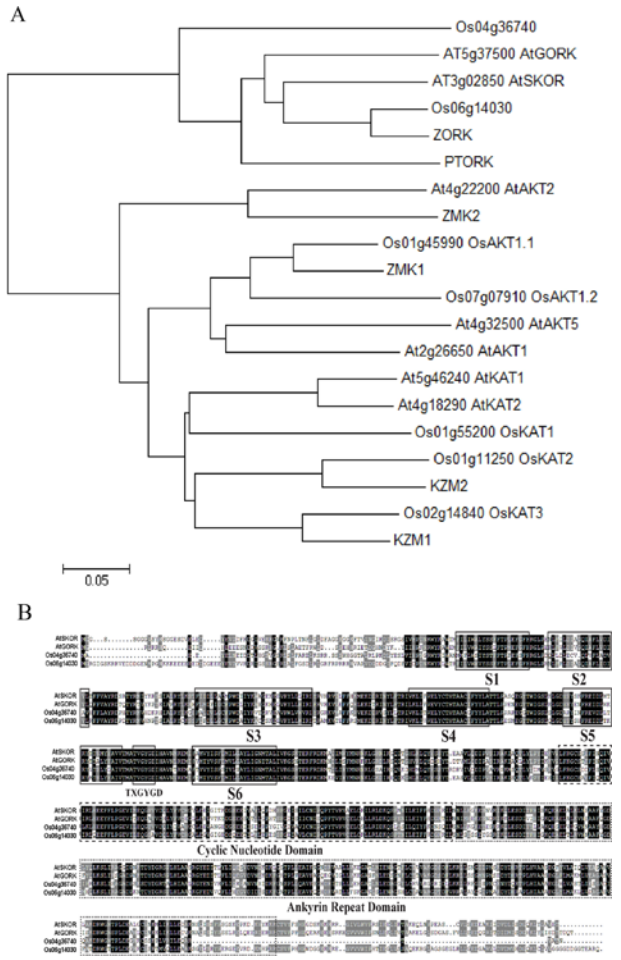

Fig. 1. Phylogenetic analysis and amino acid sequence alignment of outward-rectifying shaker-like potassium channels in rice and Arabidopsis. A. Amino acid sequences of outward-rectifying shaker-like potassium channels of Arabidopsis and rice were aligned by CLUSTALX and the tree was generated using the Mega 5.0 program by maximum likelihood method. B. The amino acid sequences of OsSKOR and OsGORK from rice and AtSKOR and AtGORK from Arabidopsis were aligned using the program CLUSTALX and then optimized manually using the GeneDoc program. Identical amino acids are shaded. The transmembrane domains (S1-S6), cyclic nucleotide binding domain and ankyrin repeat domain are indicated.

Identification of Two Rice Orthologues of Arabidopsis Outward-rectifying Potassium Channels

To identify the outward-rectifying potassium channels in rice, we used amino acid sequences of *AtSKOR* (At3g02850) and *AtGORK* (At5g37500) from *Arabidopsis thaliana* as queries in BLASTP 2.0 at the Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). Using e-value < $2e^{-15}$ as a cut-off, two genes were selected as candidate orthologues of *AtSKOR* and *AtGORK* in rice. We constructed a phylogenetic tree of the two rice predicted proteins with AtSKOR and AtGORK (Fig. 1A). Amino acid sequence alignment showed that the candidate rice outward rectifier potassium channels have the typical domains of shaker-like K^+ channels, including the conserved six-transmembrane domains, a putative cyclic nucleotide binding domain, the GYGD motif of the pore-forming region, and a C-terminal region containing an ankyrin repeat domain (Fig. 1B). Thus, these two rice genes might encode functional outward-rectifying potassium channels.

Differential Gene Expression of Two Outward-rectifying Shaker-like K^+ Channels in Rice

In Arabidopsis the biological functions of the two outward-rectifying K^+ channels were classified based on their cell-specific gene expression patterns, with *AtSKOR* expressed in root stelar cells and *AtGORK* functioning in guard cell movement and expressed in several tissues including guard cells. Accordingly, we determined the gene expression patterns of the two candidate outward-rectifying shaker-like K^+ channels from rice. Total RNAs were isolated from shoot and root of two-week-old rice seedlings and reverse transcriptase quantitative real-time PCR (qRT-PCR) was performed. Gene expression of *Os04g36740* was abundantly detected in root, but not in shoot (Fig. 2A). By contrast, *Os06g14030* was expressed in both shoot and root. To examine the gene expression patterns in more detail, we carried out qRT-PCR using RNA samples prepared from various tissues of mature rice plant including blade, sheath, node, tiller, flower, root, and seed. *Os04g36740* was expressed significantly in root (Fig. 2B). *Os06g14030* was expressed at low levels in leaf blade, leaf sheath, node, tiller and root. However, *Os06g14030* was highly expressed in flower (Fig. 2C). These data suggest that two outward shaker-like potassium channels are differentially expressed in specific tissues in rice.

We further analyzed the tissue-specific expression of *Os06g14030* and *Os04g36740* using transgenic plants harboring the *GUS* reporter gene under the control of the promoters of the two genes. In this experiment, *Os06g14030* promoter-GUS transgenic plants were not stained by X-glu (data not shown). In the case of the *Os04g36760* promoter, *GUS* staining was detected mainly in the root, flower, and seed scutellum, but was not found in the shoots of young seedlings,

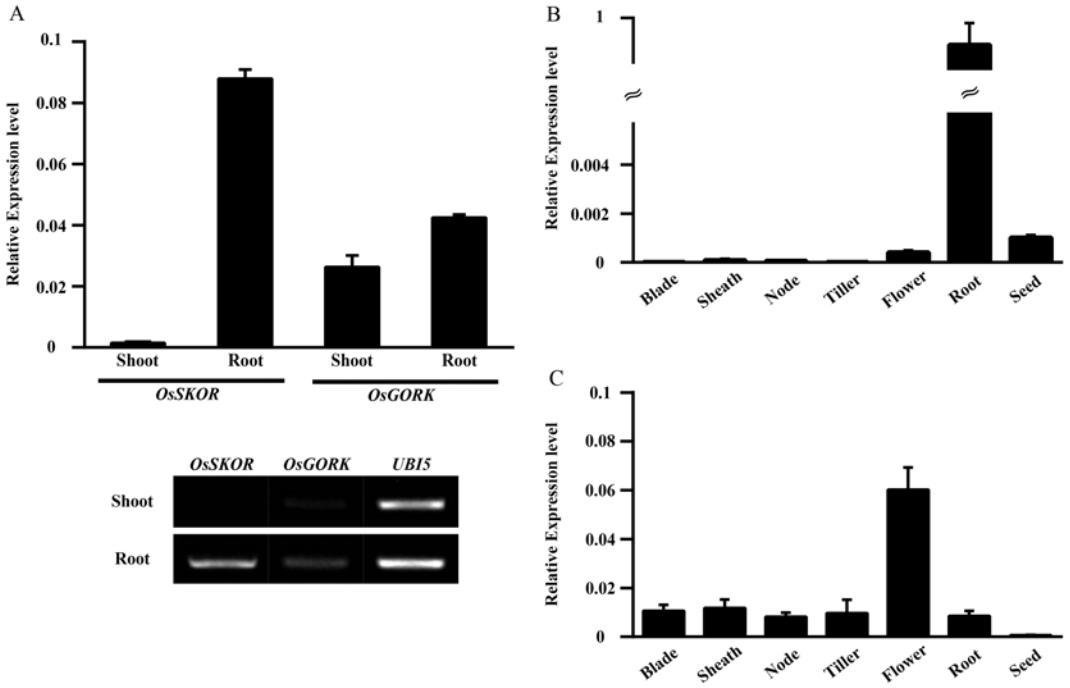

Fig. 2. Relative gene expression patterns of two outward-rectifying shaker-like potassium channels in several different tissues. (A) Real-time PCR (upper panel) and semi-quantitative RT-PCR (lower panel) were performed for *OsSKOR, OsGORK* and *UBI5* (internal control) using total RNA isolated from shoots and roots of two-week-old rice seedlings. (B and C) Real-time PCR was performed for *OsSKOR* and *OsGORK* using total RNA isolated from blade, sheath, node, tiller, flower, root and seed of mature rice crops. Data are presented as means ± standard error (SEM).

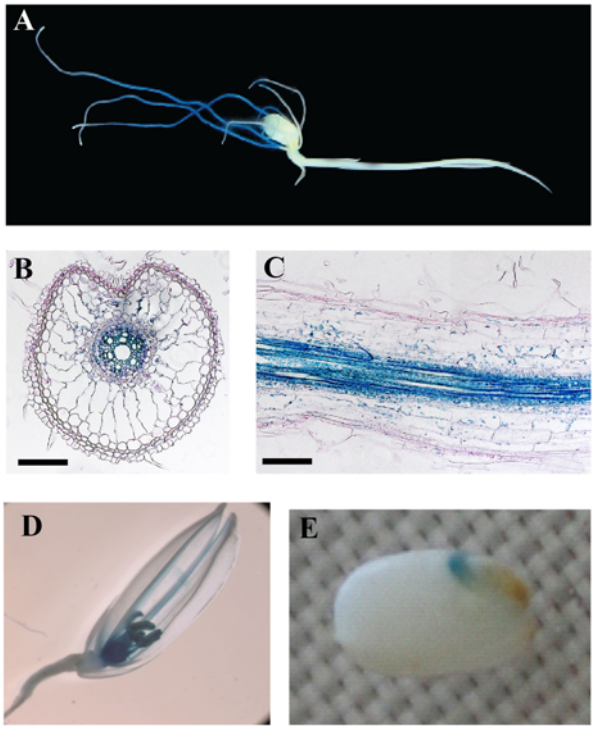

Fig. 3. *GUS* expression patterns for the *OsSKOR* promoter. **(**A) whole plant of young seedling. Paraffin sections in the horizontal (B) and longitudinal (C) orientation showed the specific expression of *GUS* in vascular cylinder. Early stage flower (D) and dried seed (E) were stained to different degrees. Scale bars indicate 50 μm.

consistent with the gene expression patterns reported by qRT-PCR (Fig. 3A). In microscopic observation of root sections of *Os04g36740* promoter-*GUS* fusion plants, most of the staining was observed in the vascular cylinder (Fig. 3A, B and C). Based on all of the above results, we named *Os04g36740* as *OsSKOR* and *Os06g14030* as *OsGORK*.

OsSKOR and OsGORK are Localized to the Plasma Membrane

GORK and SKOR function in cellular K^+ export. Accordingly these proteins should be localized on the plasma membrane. To determine the subcellular localization of OsSKOR and OsGORK in rice cells, we transiently expressed *OsSKOR:GFP* and *OsGORK:GFP* fusion genes together with pm-mCherry (plasmamembrane marker) in rice protoplasts (Nelson et al. 2007). As expected, the fusion proteins were co-localized with pm-mCherry on the plasma membrane (Fig. 4A and B). These results suggest that OsSKOR and OsGORK were mainly localized on the plasma membrane, consistent with a function as ion channels.

Discussion

K^+ is an important ion that plays roles in cellular functions

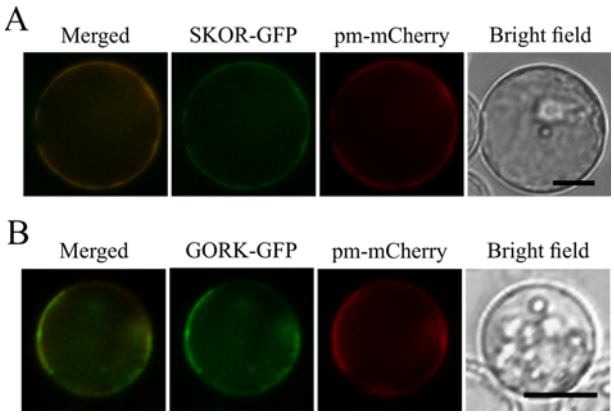

Fig. 4. Subcellular localization of OsSKOR and OsGORK. (A) OsSKOR-GFP. (B) OsGORK-GFP expressed with pm-mCherry (plasmamembrane marker). Each panel shows fluorescence and bright field images as indicated. The scale bar is 10 μm.

such as turgor adjustment, cell elongation, movement of stomata and leaves, and regulation of enzyme activity (Gierth and Maser 2007). Among stress responses, K^+ likely plays roles in Na^+ tolerance because the Na^+/K^+ ratio is the most important factor in Na^+ tolerance (Adams and Shin 2014). In addition, the outward-rectifying potassium channel GORK functions in stomatal closure and therefore is an important factor in drought tolerance (Ache et al. 2000). There are more than 77 genes encoding putative K^+ channels in Arabidopsis. Among them, 15 genes encode K^+-selective channels and the others are non-selective cation channels (NSCC). Shaker-like K^+ channels are the major gene family of K^+-selective channels (Ward et al. 2009). Previously, we identified the inward-rectifying shaker like potassium channels of rice and found that OsKAT2 is a prevalent potassium channel functioning in guard cells (Hwang et al. 2013). In this study, we found two candidate outward-rectifying potassium channels in the rice genome based on amino acid sequence similarity. However, we could not classify the rice genes based on only the phylogenic tree with *AtSKOR* and *AtGORK* of Arabidopsis because the genes encode very similar amino acid sequences and the two rice genes were not grouped separately with *AtSKOR* or *AtGORK* in the phylogenetic tree. In addition, the rice genes encode the same functional domains as found in AtSKOR and AtGORK, including cyclic nucleotide binding domain (CNBD), ankyrin repeat domain and acidic domain. However, OsGORK and OsSKOR showed conservation not only in amino acid sequence but also in gene expression pattern compared with the proteins of Arabidopsis, even though the anatomical structure of roots and stomata complexes are quite different between monocots and dicots. In Arabidopsis, *AtSKOR* is expressed in root stelar cells and *AtGORK* is expressed in guard cells. Thus, the cell-specific expression patterns of these channels might be indispensable for survival of the plant and important in their functions. Accordingly, we could classify the likely functions of the rice genes based on their gene expression patterns (Gaymard et al. 1998; Ache et al. 2000).

AKT1 is activated by phosphorylation of the C-terminal region, and a single amino acid mutation of *AtSKOR* can change the outward-rectifying potassium channel into an inward-rectifying channel (Lee et al. 2007; Li et al. 2008). SKOR and GORK proteins share the CNBD and ankyrin repeat functional domains with AKT1. The ankyrin repeat domain of the AKT1 channel is the region of interaction with CIPK23. AKT1 is activated by calcium signaling mediated by calcium sensors CBL1 and 9, and by CIPK23 interaction (Cheong et al. 2007). Calcium signaling not only activates the inward-rectifying potassium channels, but also inhibits outward-rectifying potassium channel activity. Thus, OsGORK and OsSKOR might have a similar channel activity regulation system as AKT1.

In summary, we identified *OsSKOR* and *OsGORK* based on amino acid sequence, gene expression patterns and subcellular localization in rice. These results provide valuable information to study K^+ regulation in monocots.

Materials and Methods

Plant Materials and Growth Conditions

Rice (*Oryza sativa* L. ssp. *Japonica* cv. Dongjin) was used for all experiments in this study. For sterilization, dehusked rice seeds were rinsed in 70% ethyl alcohol for 30 s and treated with 50% sodium hypochlorite for 40 min and then washed several times in sterilized water. These seeds were germinated and grown in 1/2 Murashige and Skoog (MS) medium supplemented with 0.4% phytagel and adjusted to pH 5.8 under long-day conditions with a photoperiod of 16 h light and 8 h dark at 28°C.

Cloning of Two Rice Genes for Outward-rectifier Potassium Channels

The full-length cDNA and promoters of *OsSKOR* (Os04g36740) and *OsGORK* (Os06g14030) were amplified by PCR from rice cDNA and genomic DNA. The primer sequences for PCR are listed in Table S1. The PCR products were cloned into the pENTR D-TOPO entry vector (Invitrogen, USA) prior to cloning into destination vectors.

Construction and Histochemical X-Glu Staining of Promoter-GUS Reporter Transgenic Rice

Promoters of *OsSKOR* and *OsGORK* cloned in the pENTR D-TOPO vector were introduced into the plant *GUS* reporter expression vector pBGWSF7. The resulting promoter-GUS fusion reporter constructs and the empty pBGWSF7 vector were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Rice transgenic plants were generated by the *Agrobacterium*-mediated co-cultivation method, and the transformants were screened based on phosphinothricin (PPT) resistance. For histochemical assays, transgenic plants were grown on 1/2 MS medium for young seedling staining and on soil for mature plant tissue staining. GUS staining was performed using 1 mM X-Glu buffer, which consisted of 100 mM sodium phosphate

monobasic monohydrate ($NaH_2PO_4.H_2O$), 10 mM NaEDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$) and 0.1% Triton-X 100, adjusted to pH 7.5. The transgenic plants were soaked in buffer overnight at 37°C. GUS-stained plants and tissues were fixed by washing in 70% ethanol several times until the chlorophyll was completely removed from the tissue.

Paraffin sectioning and hematoxylin and eosin (H&E) staining of rice young seedlings were performed following standard protocols. Briefly, GUS-stained plants were re-fixed overnight in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Paraffin-embedded tissues were subjected to serial 4-μm sectioning on a RM2255 microtome (Leica, Germany) in a horizontal and longitudinal orientation and stained with H&E. The collected slices were mounted with Permount (Thermo Fisher Scientific Inc., USA) and visualized using a Zeiss AxioCam MRc CCD camera and a Zeiss Axioimager M1 fluorescence microscope (Carl Zeiss, Jena, Germany).

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from shoots and roots of one-week-old rice frozen in liquid nitrogen. About 100 mg tissues were ground with a MicroSmash (Tomy, Japan), and total RNA was purified using RNeasy Plant Mini Kit (Qiagen Inc., USA). First-strand cDNA was synthesized from the total RNA using cDNA EcoDry^{TM} Premix (Clontech, Japan). Equal amounts of cDNA samples were used in amplification with 2X Power SYBR Green PCR Master mix (Biosystems, USA) in a MyiQ real time PCR system (Biorad, USA). Routinely, three replicate reactions were used for each sample. Data were calculated using C_T, defined as the PCR threshold cycle number. Relative levels of gene expression were estimated using the ΔC_T method (Livak and Schmittgen 2001). The ΔC_T value was determined by subtracting the C_T value for the endogenous control, *UBIQUITIN 5*, in each sample from that of the target. All oligonucleotides used in real time qPCR analysis are listed in supplementary Table 1.

Isolation of Rice Protoplasts and Subcellular Localization

For isolation of rice protoplasts, surface-sterilized rice seeds were grown in a darkroom for 8 d at 28ºC. The etiolated seedlings were transferred to the light and grown for 2 additional days. Leaf sheaths were chopped with a razor blade into 1-2 mm pieces and then soaked in enzyme solution [1.5% cellulose R-10 (Yakult Honsa Co., Japan), 0.75% macerozyme R-10 (Yakult Honsha Co., Japan), 600 mM mannitol, 10 mM MES, 0.1% BSA, 3.4 mM $CaCl_2$, 5 mM b-mercaptoethanol and 50 μg/mL ampicillin adjusted to pH 5.6] with gentle shaking (50-60 rpm) at 28ºC for 4-5 h. The digested samples were filtered using nylon mesh with 100-μm diameter pores and then the remnants were washed out with W5 solution (0.1% glucose, 0.9% NaCl, 2 mM MES, 0.08% KCl and 125 mM $CaCl_2$ at pH 5.65). The liberated protoplasts were collected at 100 g for 10 min at room temperature. Resuspended protoplasts were layered over 22% (w/w) sucrose solution and then centrifuged at 100 g for 10 min. The protoplast pellet was resuspended in MaMg (0.6 M mannitol, 15 mM $MgCl_2$, and 5 mM MES, adjusted to pH 5.65). Individual plasmid DNAs were introduced into rice protoplasts using the PEG-mediated method (Chen et al. 2006).

For green fluorescent protein (GFP) fusion protein expression, the *OsSKOR* and *OsGORK* full-length cDNAs in entry vector were recombined *via* LR recombination reactions into the pMDC83 vector. The resulting constructs (10 μg) were used for transfection of rice protoplasts. GFP fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Germany) with filter set XF116 (Omega, USA). The data were processed using Adobe Photoshop software (Mountain View, USA), and the images are presented in pseudocolor.

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Author's Contributions

HYK performed histochemical GUS staining and Q-PCR and wrote the manuscript. EHC performed the GFP vector construction and phylogenetic analysis. MKM performed subcellular localization experiments for OsGORK and OsSKOR-GFP. HSH cloned the *OsGORK* cDNA, *OsSKOR* cDNA and promoters of *OsGORK* and *OsSKOR*. SJM, ISY, MOB revised the manuscript and designed the experiments. BGK wrote the manuscript and designed experiments. All authors have agreed to the contents of the manuscript and declare no conflicting interests.

Supporting Information

Table S1. List of primers used for cloning, real-time PCR and RT-PCR.

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