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The rice diacylglycerol kinase family: functional analysis using transient RNA interference

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Diacylglycerol kinase (DGK) is a pivotal enzyme that phosphorylates diacylglycerol (DAG) to form phosphatidic acid (PA). The production of PA from phospholipase D (PLD) and the coupled phospholipase C/DGK route is an important signaling process in animal and plant cells. In this study, we report a genomic analysis of eight putative rice DGKs encoded by a gene family (*OsDGKs*) grouped into three clusters. To further investigate the functions of the *OsDGKs*, a double-stranded RNA (dsRNA)-induced RNA silencing method was established. Introduction of *in vitro*-synthesized dsRNAs corresponding to a unique or conserved region of *OsDGKs* into rice protoplasts abolished or diminished the expression of individual or multiple *OsDGK* genes. Suppressing the expression of *OsDGKs* resulted in a distinct depletion of the transcripts of the defense gene *OsNPR1* and the salt-responsive gene *OsCIPK15*. Our primary results suggest that *OsDGKs* are involved in the signaling of stress responses.

Keywords: diacylglycerol kinase, double-stranded RNA, rice, RNA interference

INTRODUCTION

Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] to produce diacylglycerol (DAG), which is phosphorylated to phosphatidic acid (PA) by diacylglycerol kinases (DGKs; Arisz et al., 2009). In animal cells, DAG and PA are important signaling molecules. DGK is thought to act as a conversional switch with two functional implications: the termination of DAG signaling and the initiation of PA signaling (Frere and Paolo, 2009). However, no direct DAG target (such as PKC in animal cells) has been found in plants, and the role for DAG as a plant signaling molecule has yet to been confirmed (Munnik and Testerink, 2009). In contrast, PA generated from both the PLD and PLC/DGK pathways is emerging as a stress signal molecule in plants (Munnik and Testerink, 2009; Hong et al., 2010; Zhang et al., 2010). PLD-derived PA has been found to regulate a series of developmental and environmental responses *via* its downstream targets (Li et al., 2009; Zhang et al., 2010). Relatively little genetic evidence for PA from PLC-coupled DGK regulating signaling in plant cells has been found (Munnik and Testerink, 2009).

Diacylglycerol kinase activity has been reported in several plant species, including tobacco, wheat, tomato, and *Arabidopsis* (Kamada and Muto, 1991; Lundberg and Sommarin, 1992; Wissing and Wagner, 1992; Katagiri et al., 1996; Snedden and Blumwald, 2000). Recently, multiple DGK-encoding genes have been isolated from plants (Snedden and Blumwald, 2000; Gómez-Merino et al., 2005; Chen et al., 2007). In *Arabidopsis*, *AtDGK2* and *AtDGK7* have been biochemically characterized (Gómez-Merino et al., 2004, 2005). A hydrophobic segment at the N-termini of *AtDGK1* and *AtDGK2* is necessary to target the resulting proteins to endoplasmic reticulum (ER) membranes (Vaultier et al., 2008).

In rice, pharmacological evidence indicates that PLC/DGK-mediated signaling is required for a benzothiadiazole-induced oxidative burst and hypersensitive cell death, and the transcription of one *OsDGK* is induced during this process (Chen et al., 2007). No genomic analysis of the rice *DGK* family has been reported. In this study, we report that *OsDGKs* are grouped into three clusters (I, II, and III) based on gene architecture, evolutionary relationships, and sequence identity. The transcription of *OsDGKs* was characterized using RT-PCR and real-time PCR, and their expression levels following treatment with xylanase or salt were analyzed. We established a transient double-stranded RNA (dsRNA)-induced RNA silencing assay that was used for rapid analysis of *OsDGK* functions in stress responses.

MATERIALS AND METHODS

SUSPENSION CELL CULTURES AND TREATMENTS

Rice (*Oryza sativa* L. Nipponbare ssp. *japonica*) suspension cells were initiated from embryogenic calli induced from mature rice scutella. The cells were grown in 100-ml conical flasks containing Murashige and Skoog liquid medium supplemented with 3% sucrose and 2 mg/l 2,4-D, and incubated on a rotary shaker (100–120 rpm) at 26 ± 2°C in darkness. The suspension cells were subcultured every 7 day.

Fresh suspension cells were collected by centrifugation after subculture for 4 to 5 day, and then treated with 200 μg/ml xylanase (*Trichoderma viride*; Fluka Bio-Chemika).

RICE SEEDLING GROWTH AND PROTOPLAST ISOLATION

Rice seedlings were grown in a growth room at 27°C for 2–3 weeks. Seedlings of 5–8″ high were used for protoplast isolation according to the method of Sheen (2001) with modifications. Stems, including the sheaths of young seedlings, were cut into ∼0.5–1.0 mm segments using a razor blade, and immediately placed in a Petri dish containing K3 medium (see **Table A1** in Appendix)

supplemented with 1.5% cellulase R-10 (Yakult Honsha, Japan) and 0.3% macerozyme R-10 (Yakult Honsha). The segments were vacuum-infiltrated for 1 h at 20 mmHg and digested in darkness with gentle shaking (∼40 rpm) at room temperature for about 4 h. Following the incubation, the enzyme solution was gently removed using a glass pipette, and the same volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, 5 mM glucose, pH 5.6) was added to the Petri dish for further shaking (∼80 rpm) for 1 h to release the protoplasts. Both the enzyme solution and the W5 solution were filtered through a 35- to 75-μm nylon mesh. The protoplasts were collected by centrifugation at 150 × *g* for 4 min at room temperature. The pelleted protoplasts were washed twice with W5 solution and counted under a microscope using a hemocytometer.

STRESS TREATMENTS

Protoplasts (1×10^5) were incubated in a six-well dish with each well containing 2 ml of WI culture medium (500 mM mannitol, 4 mM MES, 20 mM KCl, pH 5.6) supplemented with 150 μg/ml xylanase. For salt treatment, protoplasts (1×10^5) were incubated in modified WI culture medium containing 50 mM NaCl (400 mM mannitol, 4 mM MES, 20 mM KCl, 50 mM NaCl, pH 5.6). WI culture medium was used as a control. The protoplasts were incubated at 28˚C in darkness for the indicated times, followed by harvesting with centrifugation at 200 × *g* for 5 min.

RNA ISOLATION, RT-PCR, AND REAL-TIME PCR

Total RNAs were isolated from suspension cells or protoplasts using Trizol reagent according to the manufacturer's protocol (Takara, Japan). Reverse transcription was performed using Prime Script™RT Reagent Kit (Takara). RT-PCR conditions were as follows: denaturing at 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s, and a final extension at 72˚C for 5 min. The primers used for RT-PCR analyses are described in **Table A2** in Appendix. The *OsGAPDH* gene was amplified as an internal control.

Real-time PCR conditions were as follows: denaturing at 95˚C for 30 s, followed by 40 cycles of 95˚C for 5 s, 58˚C for 10 s, and 72˚C for 10 s, and a final extension at 72˚C for 5 min. The primers used for real-time PCR analyses are described in **Table A3** in Appendix. The expression level of the *OsActin* gene detected with actin-specific primers was used to standardize the RNA sample for each real-time PCR. The real-time PCR was performed according to the manufacturer's protocol (SYBR Premix Ex Taq™; Takara) in an ABI PRISM 7500 real-time PCR system.

IN VITRO SYNTHESIS OF dsRNA

In vitro synthesis of dsRNA was carried out according to the method of Zhai et al. (2009) with minor modifications. DNA templates were synthesized by PCR from rice cDNA and engineered to contain the minimal T7 RNA polymerase promoter sequence

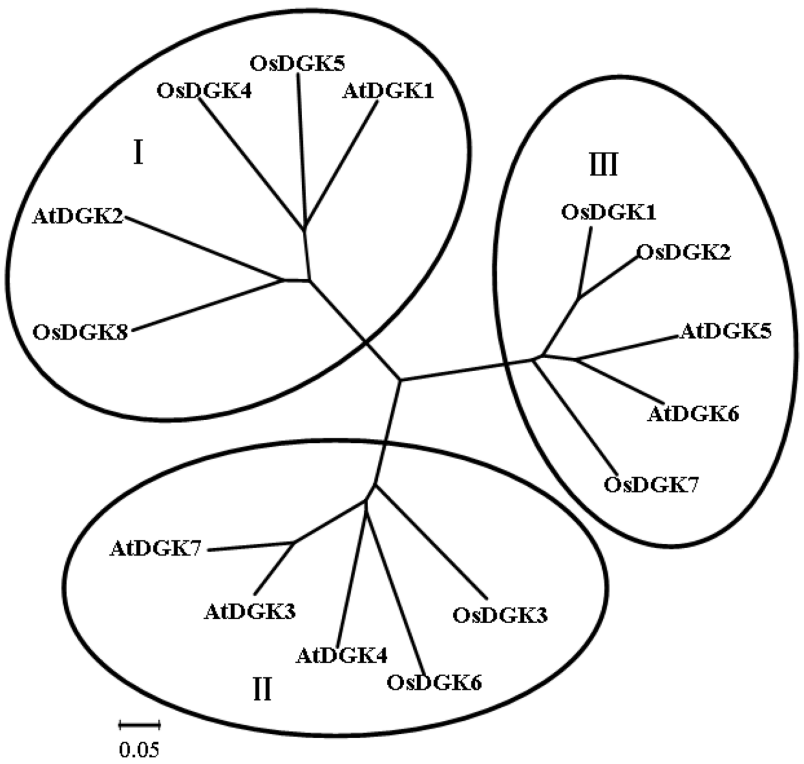

FIGURE 1 | Phylogenetic analysis of the DGK family in rice and *Arabidopsis*. Accession numbers for the DGKs are as follows: *AtDGK1* (At5g07920), *AtDGK2* (At5g63770), *AtDGK3* (At2g18730), *AtDGK4* (At5g57690), *AtDGK5* (At2g20900), *AtDGK6* (At4g28130), and *AtDGK7* (At4g30340); *OsDGK1* (Os04g54200), *OsDGK2* (Os08g08110), *OsDGK3* (Os02g54650), *OsDGK4* (Os12g38780), *OsDGK5* (Os03g31180), *OsDGK6* (Os08g15090), *OsDGK7* (Os01g57420), and *OsDGK8* (Os12g12260). At, *Arabidopsis thaliana*; Os, *Oryza sativa*. The phylogenetic analysis was performed with the neighbor-joining method using the MEGA program.

(TAATACGACTCACTATAGGGAGG) at both the 5′ and 3′ ends. The primers used to amplify DNA from the targeted genes are listed in **Table A4** in Appendix. The PCR conditions were as follows: denaturing at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. dsRNAs were synthesized *in vitro* using the RiboMAX™Large Scale RNA Production Systems T7 Kit (Promega) according to the manufacturer's recommendations. DNA templates were removed using RNase-free DNase (Promega). dsRNA was purified using the RNeasy kit (Qiagen). The dsRNA was dissolved in DEPC-treated H_2O, and its yield was measured using a UV spectrometer. Typical yields of RNA from 1 μg of DNA template were in the 80- to 100-μg range. The dsRNA was separated on a 1% agarose gel to check its integrity and size.

To prepare the fluorescent dsRNAs, a 40-bp of dsRNA directed against OsPLDα1 was synthesized, and labeled with FAM fluorescence at the 5′ end of sense strand. The sense and antisense strands of dsRNAs were: 5′-AGGCGCCACCAAGGUGUAUUCUACCA UUGAUCUGGAGAAA (sense); 5′-UUUCUCCAGAUCAAUGGU AGAAUACACCUU GGUGGCGCCU (antisense). The transfection of the dsRNA was done according to the protocol by the manufacturer (GenePharma, China). The fluorescence was visualized under a confocal microscope (TCS SP2, Leica, Germany).

TRANSFECTION OF PROTOPLASTS WITH dsRNAs

Protoplasts (1×10^6 ml^{-1}) in W5 solution were incubated on ice for 30 min. The protoplasts were pelleted and resuspended at 1×10^6 ml^{-1} in MMg solution (0.6 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.6). dsRNA (5–10 μg) was added to 100 μl of protoplasts (1×10^5 in MMg solution) to which an equal volume of PEG solution [40% (v/v) PEG4000, 0.4 M mannitol, 0.1 M $CaCl_2$] was gradually added, and the mixture was incubated at room temperature in darkness for 15 min. The transfected protoplasts were collected by centrifugation for 2 min at 150 × *g* after diluting the transfection mixture with 600 μl of W5 solution. The protoplasts were resuspended in 1 ml of W5 solution and incubated in a six-well culture plate at 28°C in darkness for the indicated times.

RT-PCR ANALYSIS OF GENE EXPRESSION IN RNAi PROTOPLASTS

At the end of the transfection, the protoplasts were collected by centrifugation for 2 min at 150 × *g*. Total RNAs were isolated from protoplasts with the Trizol reagent according to the manufacturer's protocol (Takara). Reverse transcription was performed using the Prime Script™RT reagent Kit (Takara). The primers used for RT-PCR analyses are described in **Table A2** in Appendix. The gene-silencing effect of the dsRNA was visualized in a 1% agarose gel, comparing the relative expression of RNAi-targeted genes to that of *OsGAPDH*. All photographs were taken in the Bio-Rad UV-Gel documentation system using Quantity-One analysis software.

GENOMIC SEARCH AND SEQUENCE ANALYSIS

To identify *DGK* gene homologs in rice, BLAST searches were performed using the reported sequences of *OsDGK* (Os04g54200; Zhang et al., 2008) and *AtDGK* (Gómez-Merino et al., 2004) at the TIGR[1] and NCBI[2] web sites. Sequences of rice and *Arabidopsis* DGK (Gómez-Merino et al., 2004) proteins were aligned using CLUSTAL X (ver. 1.83), and a phylogenetic tree was constructed with the neighbor-joining method using the MEGA program[3].

DOMAIN ANALYSIS

Searches for conserved domains in the OsDGK proteins were carried out using SMART[4] and PFAM[5]

RESULTS

THE DGK FAMILY IN RICE

To identify members of the DGK family in rice (*O. sativa*), sequence information from *Arabidopsis* and rice was used to perform searches of relevant DNA databases and protein domains. Text searches using the keyword "diacylglycerol kinases" were also performed. Eight putative genes were identified in rice using these approaches. The obtained sequences were further analyzed for their potential to encode a DGK using the programs PFAM and SMART. To study the evolutionary relationships between different DGK members, the MEGA program was used to analyze the phylogenetic relationships of DGKs from rice and *Arabidopsis* (**Figure 1**). The results indicate that *OsDGKs* fall into three phylogenetic clusters, as also described for the *AtDGKs* (Gómez-Merino et al., 2004). Cluster I comprises *OsDGK*(*4*, *5*, *8*), and its closest homologs are *AtDGK*(*1*, *2*); cluster II contains *OsDGK*(*3*, *6*), and *AtDGK*(*1*, *4*, *7*) are within the same group; finally, the isoforms *OsDGK*(*1*, *2*, *7*) fall into cluster III, along with *AtDGK*(*5*, *6*).

PROTEIN DOMAINS IN OSDGKs

Domain analyses using the SMART and PFAM databases revealed that the rice DGK family contains a catalytic domain, an accessory domain, a C1 domain, and a PPR domain (**Figure 2**). In eukaryotic DGKs, the kinase domain contains a conserved catalytic domain with a presumed ATP binding site, and an accessory domain to make contact with the catalytic domain (Gómez-Merino et al.,

[1]http://rice.plantbiology.msu.edu
[2]http://www.ncbi.nlm.nih.gov/
[3]http://www.megasoftware.net
[4]http://smart.embl-heidelberg.de/
[5]http://pfam.sanger.ac.uk/search.

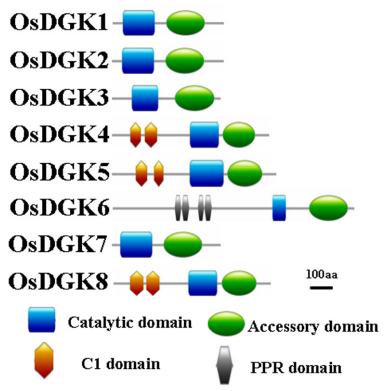

FIGURE 2 | Schematic presentation of the structure of OsDGK proteins. Conserved domains were identified by SMART and are highlighted in different colors.

2004; Arisz et al., 2009). The C1 domain contains a tandem Cys-rich sequence, which was first identified in PKC as binding to DAG and phorbol esters (PE; Azzi et al., 1992). This domain was suggested to exist in *Arabidopsis* DGKs (AtDGK1 and AtDGK2; Gómez-Merino et al., 2004; Arisz et al., 2009). The PPR (pentatricopeptide repeat) domain has been proposed to mediate macromolecular interactions. More than 400 genes encoding PPR proteins have been reported to exist in *Arabidopsis*, and most are predicted to reside in either mitochondria or chloroplasts (Small and Peeters, 2000; Lurin et al., 2004).

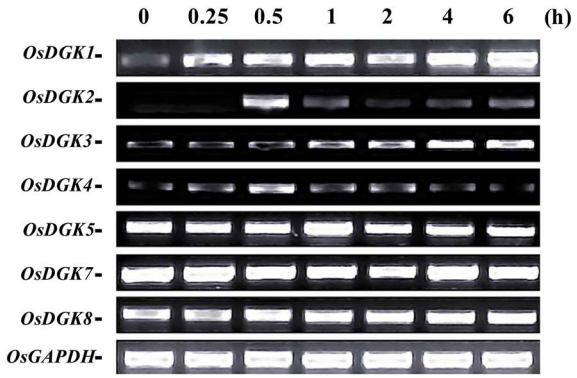

FIGURE 3 | RT-PCR analysis of *OsDGK* transcripts in rice cells treated with xylanase. Suspension cells were treated with 200 μg/ml xylanase for the indicated times, and *OsDGK* transcripts were monitored by RT-PCR. Approximately 0.1 μg of total RNA was used in each PCR. *OsDGK6* was undetectable in cells with or without xylanase treatment. The *OsGAPDH* gene was used as an internal control.

All of the *OsDGKs* were found to contain a catalytic domain and an accessory domain. In addition, *OsDGK*(*4*, *5*, *8*) harbor two C1 domains, whereas *OsDGK6* has four putative PPR domains (**Figure 2**). To our knowledge, among the reported plant DGKs, only *OsDGK6* contains a PPR domain. It should be noted that *OsDGK6* could be a unique (or putative) *OsDGK*, according to protein structure analysis (**Figure 2**), but it was nevertheless grouped into the same cluster (II) as *OsDGK3* in a bioinformatics analysis (**Figure 1**). A similarity analysis revealed the highest similarity between *OsDGK6* and *OsDGK3* (**Table A5** in Appendix). In addition, we isolated all *OsDGK* cDNAs except for that of *OsDGK6*. RT-PCR analysis did not detect the *OsDGK6* transcript under normal or stress conditions (**Figure 3**); therefore, the existence and function in rice of this *OsDGK* have yet to be determined.

XYLANASE-INDUCED EXPRESSION OF *OsDGK* IN CELLS

The expression of *OsDGK* genes in suspension cells was analyzed using RT-PCR. Under control conditions, the transcription of six of the eight *OsDGKs* (all except for *OsDGK2* and *OsDGK6*) was confirmed. The activation of PLC and DGK pathway has been reported in tomato cells treated with the fungal elicitor xylanase (Laxalt et al., 2007). We therefore explored whether

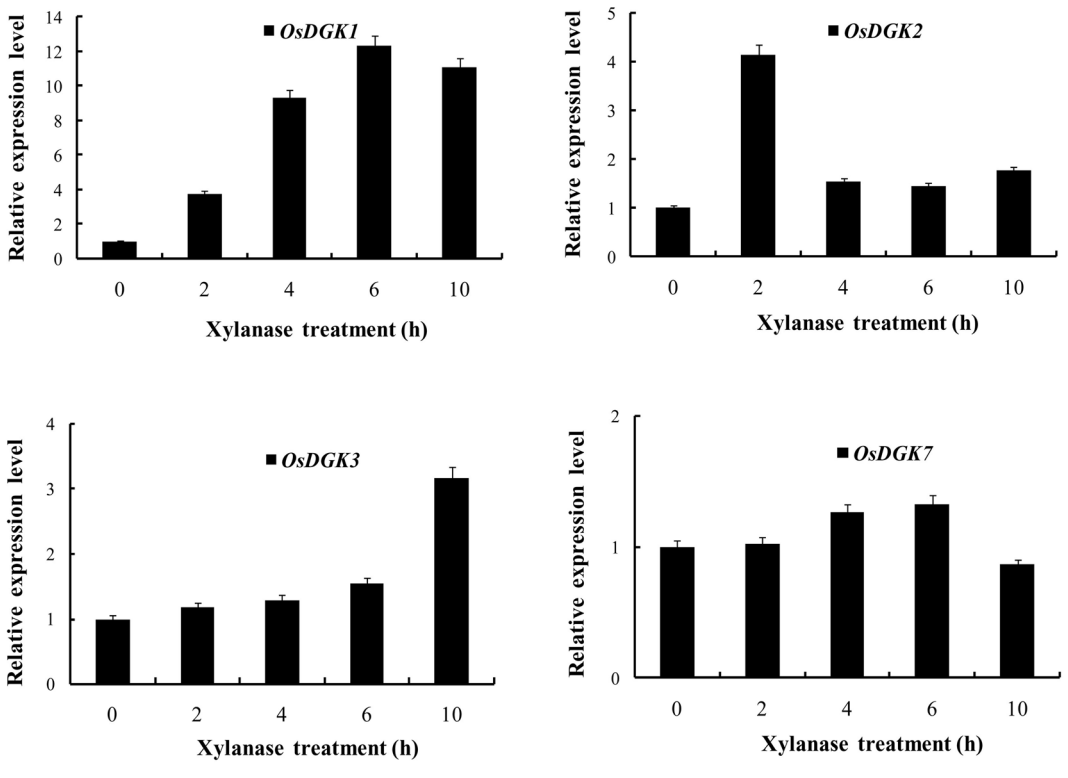

FIGURE 4 | Real-time PCR analysis of the expression of *OsDGK*(*1, 2, 3, 7*). Rice protoplasts were treated with 150 μg/ml xylanase. The transcripts of four *OsDGK* genes were monitored with real-time PCR. The data shown are averages of three replicates, each with three PCR samples from the same cDNA archive.

expression of *OsDGK(s)* are activated by the xylanase. Treatment of cells with 200 μg/ml xylanase led to increases in the transcription of *OsDGK*(*1*, *2*, *3*, *4*). Although the basal transcript levels of *OsDGK*(*5*, *7*, *8*) were high, their transcription was not affected by xylanase treatment. The transcription of *OsDGK6* was undetectable with or without xylanase (**Figure 3**).

We selected *OsDGK*(*1*, *2*, *3*), which were induced by xylanase, and *OsDGK7*, which was not induced by xylanase, for further testing with real-time PCR. As shown in **Figure 4**, the transcription of both *OsDGK1* and *OsDGK3* gradually increased in xylanase-treated protoplasts. The transcription of *OsDGK2* exhibited a sharp peak at 2 h after the addition of 150 μg/ml xylanase. A peak in the *OsDGK2* transcript was also found in the RT-PCR analysis, but it appeared earlier, probably due to the higher concentration of xylanase (150 μg/ml there) used. The transcription of *OsDGK7* was not affected by xylanase under the tested conditions.

TRANSIENT SUPPRESSION OF *OsDGK* EXPRESSION BY INTRODUCTION OF THE CORRESPONDING dsRNA

To study the functions of *OsDGKs* in rice cells, we established methods for dsRNA-induced RNA silencing according to published procedures (Endo et al., 2008; Zhai et al., 2009). Transfection with *in vitro*-synthesized dsRNAs against target genes into cells resulted in the depletion of their transcripts due to the combined actions of the DICER enzyme and the RNA-induced silencing complex (RISC) enzyme (**Figure 5A**).

We first selected *OsPLDα1* to test because the expression of its mRNA was characterized in our previous work (Shen et al.,

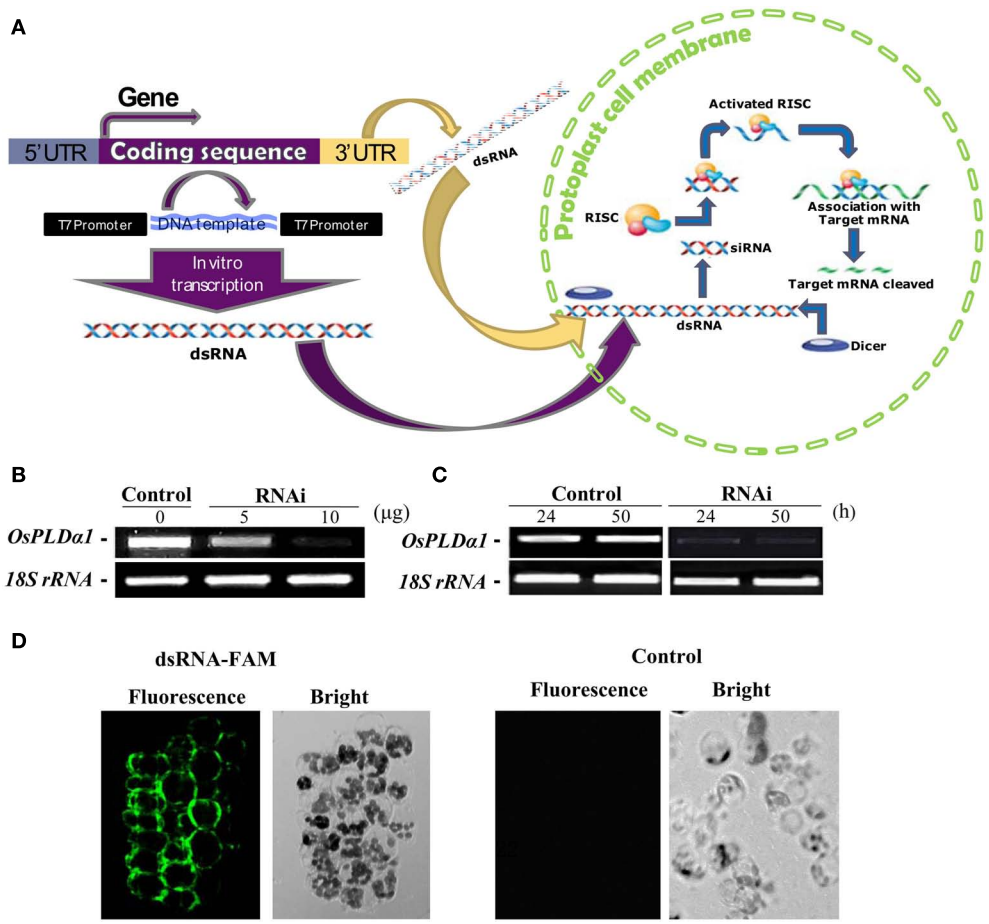

FIGURE 5 | Double-stranded RNA-induced RNA silencing in rice protoplasts. (A) Simple illustration of dsRNA-induced RNA silencing. When a long dsRNA is introduced into plant cells, the endogenous DICER enzyme cuts long dsRNA into short siRNAs. The RNA-induced silencing complex (RISC) enzyme attaches to these siRNAs, forming the siRNA–RISC complex (Plasterk, 2002). The target mRNA is attached to the siRNA–RISC complex and cleaved into small pieces. This process inhibits the expression of target genes. **(B)** Dose dependence of the transient RNAi silencing of *OsPLDα1* in rice protoplasts. Protoplasts were transfected with 0, 5, or 10 μg of dsRNA against *OsPLDα1* (RNAi) or with sterile water (Control), followed by incubation in darkness for 24 h. The silencing effect of *OsPLDα1* was monitored using RT-PCR. **(C)** Time dependence of transient RNAi silencing of *OsPLDα1* in rice protoplasts. Protoplasts were transfected with 10 μg of a dsRNA against *OsPLDα1* (RNAi) or with sterile water (Control). RNAi and control protoplast samples were collected at 24 and 50 h, respectively, and then analyzed with RT-PCR for *OsPLDα1* transcripts. *18S rRNA* was amplified as an internal control. **(D)** A 40-bp of synthesized dsRNA labeled with FAM fluorescence at the 5′ end of sense strand against *OsPLDα1* was transfected into rice protoplasts. The fluorescence was visualized under a confocal microscope after 8 h incubation in darkness. Control, protoplasts were transfected with water.

2011). A dsRNA against a 486-bp sequence corresponding to the *OsPLDα1* coding sequence was synthesized *in vitro*. Rice protoplasts were transfected with 5 or 10 μg of dsRNA (RNAi) or with sterile water (control, as a mock transfection) and incubated for 24 h in darkness. The silencing effects of dsRNA were dose-dependent (**Figure 5B**). This inhibition of transcription lasted for at least 50 h (**Figure 5C**).

To prove the *in vitro*-synthesized dsRNA was transported into protoplasts, we synthesized a 40-bp dsRNA labeled with FAM fluorescence at the 5′ end of sense strand against *OsPLDα1*, and transfected into rice protoplasts. The transfected protoplasts were visualized under a fluorescent microscope after 8 h incubation in darkness. The fluorescence was found in protoplasts. As a control, no fluorescence was found in the protoplasts transfected with sterile water (**Figure 5D**). This together with the RT-PCR results suggest that *in vitro*-synthesized dsRNA had been transported into protoplasts and suppressed gene transcripts.

We then tried to silence expression of the *OsDGK* family. Two *in vitro*-synthesized dsRNAs against conserved regions of *OsDGK*(*1*, *2*, *3*, *7*) and *OsDGK*(*4*, *5*, *8*) were simultaneously transfected into protoplasts. Mock-transfected protoplasts (control) and two dsRNA-transfected protoplasts (RNAi) were collected for RT-PCR analysis after incubation in darkness for 24 h. The abundance of the *OsDGK* transcripts after RT-PCR analysis was normalized to the internal standard gene *OsGAPDH*. As shown in **Figure 6A**, the transcription of seven *OsDGKs* was successfully repressed simultaneously by two dsRNAs as compared the mock-transfected control.

We next carried out gene-specific interference of the expression of *OsDGK*s. RT-PCR analysis was undertaken for *OsDGK2*, *OsDGK3*, and *OsDGK7* after transient RNAi using *in vitro*-synthesized dsRNAs corresponding to their 3′-untranslated regions (3′-UTRs). The expression of *OsDGK2* was repressed significantly by introducing *in vitro*-synthesized dsRNA directed against its 3′-UTR, whereas the expression of *OsDGK3* and *OsDGK7* was not affected (**Figure 6B**). Similarly, gene-specific interference was found for *OsDGK3* versus *OsDGK2* and *OsDGK7*, and *OsDGK7* versus *OSDGK2* and *OsDGK3*. Taken together, these results suggest that the introduction of dsRNAs is effective for targeted gene silencing in rice protoplasts.

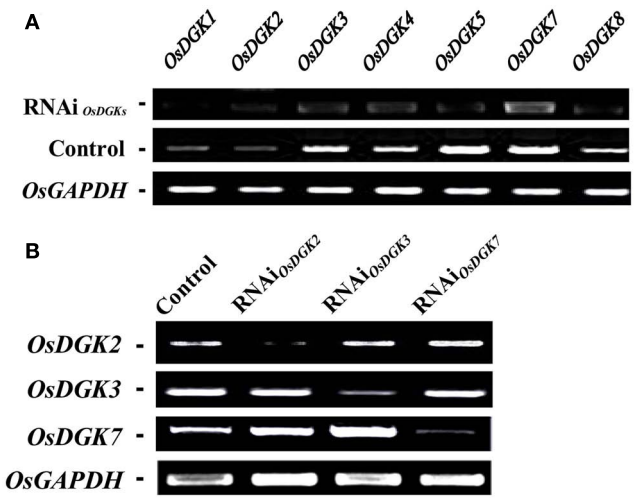

FIGURE 6 | Multi-gene and specific gene silencing of *OsDGKs* in protoplasts using transient RNAi. (A) Multi-gene silencing of *OsDGKs* in rice protoplasts using transient RNAi. RT-PCR analysis of *OsDGKs* was performed after transient RNAi by simultaneously introducing two *in vitro*-synthesized dsRNAs against conserved regions of *OsDGK*(*1, 2, 3, 7*) and *OsDGK*(*4, 5, 8*). Mock-transfected (Control) and two dsRNA-transfected protoplasts (RNAi) were incubated for 24 h, followed by protoplast collection for RNA isolation. *OsGAPDH* was amplified as an internal control. **(B)** Gene-specific silencing of *OsDGKs* in rice protoplasts with transient RNAi. RT-PCR analysis of *OsDGK2, OsDGK3,* and *OsDGK7* was performed after transient RNAi using *in vitro*-synthesized dsRNAs against the 3′-UTR regions of each. dsRNA-transfected (RNAi) and mock-transfected (Control) protoplasts were incubated for 24 h, followed by protoplast collection for RT-PCR analysis. *OsGAPDH* was amplified as an internal control.

EFFECT OF THE TRANSIENT SILENCING OF *OsDGK* ON THE TRANSCRIPTION OF GENES RELATED TO STRESS TOLERANCE

Using the transient RNA interference assay, we next explored the functions of the *OsDGK*s in stress responses. The *WRKY* transcriptional factors have been reported to be involved in various stress responses; in particular, overexpression of *OsWRKY71* enhances resistance to virulent bacterial pathogens (Liu et al., 2007). Treatment of protoplasts with xylanase (150 μg/ml) induced an increase in the expression of *OsWRKY71* and the pathogen-related gene *OsNPR1* (Liu et al., 2005). Transient silencing of *OsDGK*s triggered by the introduction of two dsRNAs corresponding to the conserved regions of *OsDGK*(*1*, *2*, *3*, *7*) and *OsDGK*(*4*, *5*, *8*; **Figure 6A**) prevented the increases in expression of *OsWRKY71* and *OsNPR1* induced by xylanase (**Figures 7A,B**).

We next asked whether *OsDGKs* regulate abiotic stress responses. The expression of *OsWRKY7* was not affected by salt stress; transient silencing of *OsDGK*s had no effect on *OsWRKY7* expression (**Figure 8A**). However, under salt stress, the expression of *OsCIPK15* (CIPK, for calcineurin B-like protein interaction protein kinase) was induced, and the overexpression of *OsCIPK15* improved the salt tolerance of rice seedlings (Xiang et al., 2007). An increase in the transcription of *OsCIPK15* was also observed in rice protoplasts exposed to NaCl solution. This NaCl-induced increase in *OsCIPK15* was repressed in cells in which *OsDGK*s were transiently silenced (**Figure 8B**). These results suggest that *OsDGKs* regulate abiotic and biotic stresses through different signaling pathways.

DISCUSSION

Diacylglycerol kinase phosphorylates DAG to generate PA, serving as a DAG consumer as well as a PA generator. Therefore, DGK is thought to regulate the balance between these two lipid messengers by catalyzing their interconversion (Frere and Paolo, 2009). The mammalian DGKs are a large enzyme family with 10 isozymes, which are subdivided into five groups according to their structural features (Sakane et al., 2007). In the *Arabidopsis* genome, seven DGK isoforms that form three clusters have been identified (Gómez-Merino et al., 2004). The rice DGKs also group into three clusters (**Figure 1**). Both *Arabidopsis* and rice DGKs contain catalytic and accessory domains (Gómez-Merino et al., 2004; **Figure 2**). OsDGK(4, 5, 8) and AtDGK(1, 2) belong

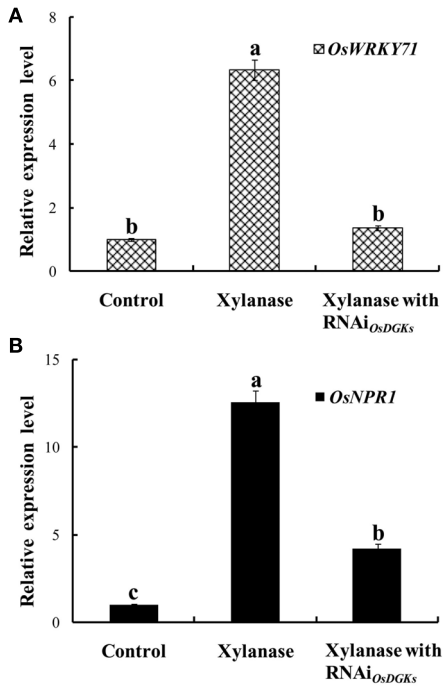

FIGURE 7 | Effects of silencing of *OsDGKs* on *OsWRKY71* and *OsNPR1* transcription induced by xylanase in rice protoplasts. After transient RNAi using two *in vitro*-synthesized dsRNAs against conserved regions of *OsDGK*(*1, 2, 3, 7*) and *OsDGK*(*4, 5, 8*) for 24 h, the transfected protoplasts were treated with 150 μg/ml xylanase for 6 h. The protoplasts were collected for real-time PCR analysis to detect transcripts of *OsWRKY71* **(A)** and *OsNPR1* **(B)**. Values followed by different letters differ significantly ($P < 0.01$).

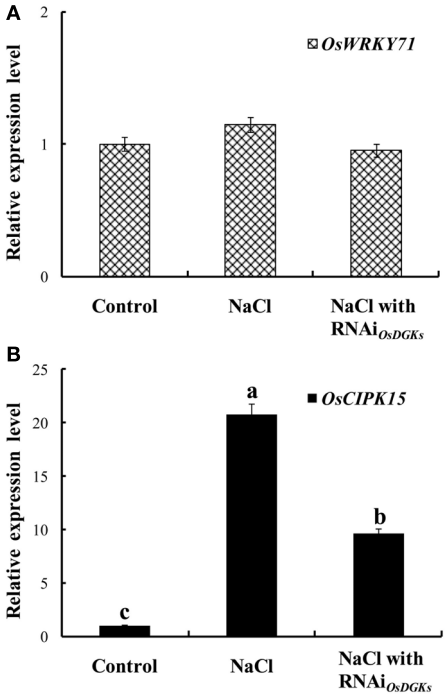

FIGURE 8 | Effects of silencing of *OsDGKs* on transcription of *OsWRKY71* and *OsCIPK15* induced by NaCl in rice cells. Following transient RNAi using two *in vitro*-synthesized dsRNAs against conserved regions of *OsDGK*(*1, 2, 3, 7*) and *OsDGK*(*4, 5, 8*) for 24 h, the transfected protoplasts were treated with 50 mM NaCl for 6 h. The protoplasts were collected for real-time PCR analysis to detect transcripts of *OsWRKY71* **(A)** and *OsCIPK15* **(B)**. Values followed by different letters differ significantly ($P < 0.01$).

to cluster I, which possesses two cysteine-rich domains conferring C1 domains. These domains are absent from other *Arabidopsis* and rice DGKs (**Figure 2**; Vaultier et al., 2008). The C1 domain can bind to proteins to modulate the interaction with lipids and proteins (Colon-Gonzalez and Kazanietz, 2006). AtDGK7 still displays kinase activity in the absence of the C1 domain (Gómez-Merino et al., 2005), suggesting that this domain is not necessary for its phosphorylation activity (Snedden and Blumwald, 2000). By fusion with fluorescent proteins, the hydrophobic segment in the amino-terminal region upstream of the C1 domain in AtDGK1 and AtDGK2 has been proven to be sufficient and necessary to sequester proteins to ER membranes (Vaultier et al., 2008). However, whether the final localization of full-length DGKs is identical with this segment is unclear. Much less is known about plant DGKs than animal DGKs.

Transient RNA interference caused the decreased expression of unique and multiple *OsDGK* genes in rice protoplasts (**Figure 6**). This approach has also been successfully used in *Arabidopsis* and *Zinnia* (Endo et al., 2008; Zhai et al., 2009). Introduction of *in vitro*-synthesized dsRNAs corresponding to the cellulose synthesis gene *CesA* into *Zinnia* cells repressed the expression of *Zinnia CesA* homologs. The repression phenocopies *Arabidopsis* cellulose synthase mutants that have defects in secondary cell wall synthesis and increased abnormal tracheary elements (Endo et al., 2008). In our work, repression of multiple *OsDGK* genes impaired stress-induced gene expression (**Figures 7** and **8**). The combination of transient RNA interference and *DGK* mutants will help to fully elucidate the functions of DGKs in plants.

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APPENDIX

Table A1 | K3 medium used for rice protoplast system.

K3 medium	10 × B5 Macro, 100 × B5 micro (I), 1000 × B5 micro (II), 100 × B5 vitamins, 200 × MES (0.1 g/ml), 500 × myo-inositol (0.05 g/ml), 100 × NH_3NO_3 (25 mg/ml), 100 × $CaCl_2$ (75 mg/ml), 100 × xylose (25 mg/ml), 0.4 M d-mannitol. pH is adjusted to 5.6 by 1 M KOH.
10 × B5 Macro (1 l)	KNO_3 25 g, $(NH_4)_2SO_4$ 1.34 g, $CaCl_2 \cdot 2H_2O$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, $NaH_2PO_4 \cdot H_2O$ 1.5 g.
100 × B5 Micro(I) (1 l)	$MnSO_4 \cdot H_2O$ 0.78 g, $ZnSO_4 \cdot 7H_2O$, 0.2 g, H_3BO_3 0.3 g, KI 0.075 g.
1000 × B5 Micro(II) (1 l)	$NaMoO_4 \cdot 2H_2O$ 0.250 g, $CuSO_4 \cdot 5H_2O$ 25 mg, $CoCl_2 \cdot 6H_2O$ 25 mg.
100 × B5 Vitamins (1 l)	Vitamin B1 (thiamine-HCl) 1 g, vitamin B6 (pyridoxine-HCl) 0.1 g, nicotinic acid 0.1 g.

K3 medium was prepared according to the method by Chen et al. (2006) with minor modification.

Table A2 | List of oligos used for RT-PCR analysis.

Genes	Directions	Oligos (5′–3′)
OsDGK1	Forward	CTGGCACCAGGAAAGTACAAGATAGAGAC
	Reverse	TCGTGGTTGCTACAGCACATCGG
OsDGK2	Forward	AGACTTATTGAGGTTGTTGGATTCCGTGAT
	Reverse	CAGGGATCTTGAATGTATCTGCGGC
OsDGK3	Forward	GTTCTGAATGGGAGCAAGTTACAATGC
	Reverse	AGAGAAGGGTAAGGAACTTTGTTTATCTCG
OsDGK4	Forward	ATCGCTCTGAGGAGGATTCTTTCTGC
	Reverse	CTTCAATATCAGATGGCGGGTCAATAG
OsDGK5	Forward	TGAGATTCCAGAGGATTCAGAAGGTGTT
	Reverse	CCTCTTCTGAGATGCAGTGATTAGGTGAC
OsDGK6	Forward	CCATTCGGATAGTCAAGAACCTC
	Reverse	CCAACTATGCGGACTTAACCAG
OsDGK7	Forward	GGGGAAGAGAAATCCTGGAACAGATG
	Reverse	ATTGGATGACATAGGGATGCACAGAAC
OsDGK8	Forward	TCTGTCTGTGAAAGAAGTTGCCCAAG
	Reverse	TCTTGCCGTTAATGAAAACAAGCAGT
OsPLDα1	Forward	GGTAACCGTGAGGTGAAGCA
	Reverse	GCATTCCCAGGTGCTCGTAC
OsGAPDH	Forward	ACCACAAACTGCCTTGCTCC
	Reverse	ATGCTCGACCTGCTGTCACC
18S rRNA	Forward	CCTATCAACTTTCGATGGTAGGATA
	Reverse	CGTTAAGGGATTTAGATTGTACTCATT

Table A3 | List of oligos used for real-time PCR analysis.

Genes	Directions	Oligo (5′–3′)
OsDGK1	Forward	GGCTGCTTGGTGTAGTTAGTG
	Reverse	TCTTGGTCAGTGGTTGGGTT
OsDGK2	Forward	TCGTCTGTCTCAACCTGCCTAG
	Reverse	CACGGAATCCAACAACCTCAAT
OsDGK3	Forward	GAATGGGAGCAAGTTACAATG
	Reverse	ATCGGAATGAGCTTCGACAA
OsDGK7	Forward	TACAGTCAGTAAGACAAGCGAAAG
	Reverse	CAGCGAATGAGGCAAATCCA
OsWRKY71	Forward	CGCCGACCCATCCGACCTCA
	Reverse	TCTTGACAGGGCAGGCGGGA
OsNPR1	Forward	CCCGCGATGTTCGAACGTGC
	Reverse	CGACGAGAGCCCCGACCTGT
OsCIPK15	Forward	GTTACCACTTCCTATCATATCATC
	Reverse	CTAAACATCAACTCTCCAAATAC
OsActin	Forward	AGGAAGGCTGGAAGAGGACC
	Reverse	CGGGAAATTGTGAGGGACAT

Table A4 | List of oligos used in amplifying DNA templates for *in vitro* dsRNA synthesis.

Targeted Gene	Directions	Oligos (5′–3′)	Size of dsRNA (bp)
OsPLDα1	Forward	GCTTAATACGACTCACTATAGGGAGGTTGACGATGAGTACATCATCATCGG	486
	Reverse	GCTTAATACGACTCACTATAGGGAGGCTATGAGGTGAGGATGGGGGGCATG	
OsDGKs(1,2,3,7)	Forward	GCTTAATACGACTCACTATAGGGAGGTGTCGGCTTTCGCGATGCCT	208
	Reverse	GCTTAATACGACTCACTATAGGGAGGGGCTGCTTCCATGGCTCCCC	
OsDGKs(4,5,8)	Forward	GCTTAATACGACTCACTATAGGGAGGCGCGCGCAGAGGTTAGCTCA	229
	Reverse	GCTTAATACGACTCACTATAGGGAGGGGAGGCCGCATGGCCGATAG	
OsDGK2 (3′-UTR)	Forward	GCTTAATACGACTCACTATAGGGAGGAATTGGTATCTTTTCTAGGTTGCAT	260
	Reverse	GCTTAATACGACTCACTATAGGGAGGTCTGCTGAACAATAAACAAGAAATC	
OsDGK3 (3′-UTR)	Forward	GCTTAATACGACTCACTATAGGGAGGAATCAGGGTCGTATTCTAGATCGTT	303
	Reverse	GCTTAATACGACTCACTATAGGGAGGCATAACAGGAGGGGAAATCTGAGTA	
OsDGK7 (3′-UTR)	Forward	GCTTAATACGACTCACTATAGGGAGGTGACGAGGTTTTGTACGTATGGCTG	342
	Reverse	GCTTAATACGACTCACTATAGGGAGGCGTGGAGGTATATTCTGCGGGTAGT	

Table A5 | Amino acid sequence identities among OsDGKs.

	OsDGK1	OsDGK2	OsDGK3	OsDGK4	OsDGK5	OsDGK6	OsDGK7	OsDGK8
OsDGK1		81	32	27	25	29	63	25
OsDGK2			31	25	25	29	62	24
OsDGK3				24	25	54	33	26
OsDGK4					59	17	25	50
OsDGK5						16	23	52
OsDGK6							30	17
OsDGK7								24
OsDGK8								

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