**Arabidopsis** AtDGK7, the Smallest Member of Plant Diacylglycerol Kinases (DGKs), Displays Unique Biochemical Features and Saturates at Low Substrate Concentration

**THE DGK INHIBITOR R59022 DIFFERENTIALLY AFFECTS AtDGK2 AND AtDGK7 ACTIVITY IN VITRO AND ALTERS PLANT GROWTH AND DEVELOPMENT**

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Diacylglycerol kinase (DGK) regulates the level of the second messenger diacylglycerol and produces phosphatidic acid (PA), another signaling molecule. The *Arabidopsis thaliana* genome encodes seven putative diacylglycerol kinase isozymes (named AtDGK1 to −7), structurally falling into three major clusters. So far, enzymatic activity has not been reported for any plant Cluster II DGK. Here, we demonstrate that a representative of this cluster, AtDGK7, is biochemically active when expressed as a recombinant protein in *Escherichia coli*. AtDGK7, encoded by gene locus At4g30340, contains 374 amino acids with an apparent molecular mass of 41.2 kDa. AtDGK7 harbors an N-terminal catalytic domain, but in contrast to various characterized DGKs (including AtDGK2), it lacks a cysteine-rich domain at its N terminus, and, importantly, its C-terminal DGK accessory domain is incomplete. Recombinant AtDGK7 expressed in *E. coli* exhibits Michaelis-Menten type kinetics with 1,2-dioleoyl-sn-glycerol as substrate. AtDGK7 activity was affected by pH, detergents, and the DGK inhibitor R59022. We demonstrate that both AtDGK2 and AtDGK7 phosphorylate diacylglycerol molecular species that are typically found in plants, indicating that both enzymes convert physiologically relevant substrates. AtDGK7 is expressed throughout the *Arabidopsis* plant, but expression is strongest in flowers and young seedlings. Expression of AtDGK2 is transiently induced by wounding. R59022 at ~80 μM inhibits root elongation and lateral root formation and reduces plant growth, indicating that DGKs play an important role in plant development.

Lipid second messengers, generated in response to diverse stimuli through the activity of lipid kinases and phospholipases, are involved in a variety of biological responses in plant cells (1, 2). Diacylglycerol kinase (EC 2.7.1.107) is a lipid kinase that phosphorylates diacylglycerol (DAG) to yield phosphatidic acid (PA) in a reaction that uses ATP as phosphate donor. In plants, both DAG and PA may have signaling functions. DAG has been demonstrated to activate both ion pumping in patch-clamped guard cell protoplasts and opening of stomata (3), whereas PA accumulates in response to different kinds of stresses and regulates the activity of several enzymes (4).

Various PA targets have been identified in plants, including, for example, *Arabidopsis* phosphoinositide-dependent protein kinase 1 (5) and a calcium-dependent protein kinase from carrot (6). Recently, using PA affinity chromatography coupled to mass spectrometry, PA-binding proteins were identified in plants, including phosphaenolpyruvate carboxylase, Hsp90, 14–3–3 proteins, and others (7), providing new entries into the field of plant PA research. Furthermore, Zhang et al. (8) have demonstrated that PA interacts with ABI1 phosphatase 2C, thereby regulating abscisic acid signaling in *Arabidopsis thaliana*.

Animal DGKs have been extensively studied, and models for their function have been elaborated (9, 10). DGK activity has also been reported in several plant species, including *Catharanthus roseus*, tobacco, wheat, tomato, and *Arabidopsis* (11–16), and molecular data bases reveal that they are present in a number of other crop plants such as rice, maize, grape, sweet orange, and cotton. However, functional analysis of DGK genes is still fragmentary. Two DGK cDNAs, *LeDGK1* and *LeCBDGK*, respectively, have been cloned from tomato (15). The two enzymes are derived from the same gene via alternative splicing and are identical except for a 29-amino acid-long C-terminal extension in

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5 The abbreviations and trivial names used are: DAG, diacylglycerol; EST, expressed sequence tag; Bis-Tris, bis(2-hydroxyethyl)iminoen-tris(hydroxymethyl)methane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Na-DC, sodium deoxycholate; CHAPS, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate; 1,2-SAG, 1-stearoyl-2-arachidonoyl-sn-glycerol; 1,2-DOG, 1,2-dioleoyl-sn-glycerol; 1,2-POG, 1-palmitoyl, 2-oleoyl-sn-glycerol; 1,2-SLG, 1-stearoyl, 2-linoleoyl-sn-glycerol; 1,2-OPG, 1,2-1-oleoyl, 2-palmitoyl-sn-glycerol; PA, phosphatidic acid; CaM, calmodulin; RT, reverse transcription; R59022, 6-[2-(4-[14]Fluorophenyl)phosphinylmethy- ene]-1-piperidinyl(ethyl)-7-methyl-5H-thiazolo[3,2-a]pyrimidine-5-one; R59949, 3-[2-4-[bis-(4-fluorophenyl)methyl]-1-piperidinyl(ethyl)-2,3-dihydro-2-thioxo-4(1H)-quinazolinone; MPSS, massively parallel signature sequencing; TPM, transcripts/million; HPLC, high pressure liquid chromatography.
the case of LeCBDGK, which represents a calmodulin (CaM)-binding domain. The two proteins lack the cysteine-rich domain (CRD) present in other eukaryotic DGKs, but are active in vitro. LeCBDGK is found both in association with membranes and in soluble cell extracts. The Ca\(^{2+}\)/CaM-dependent translocation of LeCBDGK to its membrane-associated substrate DAG might represent a means of activation in vivo, analogous to the Ca\(^{2+}\)/CaM-dependent translocation of mammalian DGKs. In contrast, LeDGK1 only associates with the membrane fraction, via a Ca\(^{2+}\)/CaM-independent mechanism, which might represent a means of encoding specificity in cellular responses by alternative splicing (15). In A. thaliana, seven candidate genes (named AtDGK1 to -7) encode putative DGK isoforms. The AtDGK1 cDNA has been isolated and reported to be mainly expressed in roots, shoots, and leaves, but its enzyme product was not active in vitro (17). We have previously cloned the AtDGK2 cDNA and demonstrated that its encoded enzyme is catalytically active. AtDGK2 transcripts are found in the whole plant except in stems and are induced by exposure to 4 °C, pointing to a role in cold signal transduction (18). AtDGK1 and -2 share a similar domain organization and fall into the Cluster I of plant DGKs.

Here we report the molecular cloning and characterization of AtDGK7, which encodes an enzyme structurally belonging to the Cluster II of plant DGKs. The AtDGK7 gene is transcribed throughout the plant and most prominently in flowers and young tissues. Recombinant AtDGK7 enzyme is catalytically active and, importantly, accepts DAG as a substrate. Recombinant AtDGK2 in vitro. It also modifies root growth within the same concentration range, indicating the involvement of this enzyme in developmental processes. In contrast, AtDGK7 was found to be affected by R59022 only at concentrations above 100 μM.

**EXPERIMENTAL PROCEDURES**

**General**—Manipulation and analysis of nucleic acids were performed according to standard molecular-biological techniques described (19). Restriction enzymes were purchased from Roche Applied Science (Mannheim, Germany) and subcloned into pGEM-T Easy (Promega, Madison, WI). DNA sequencing was performed by SeqLab (Göttingen, Germany) or Eurogentec (Cologne, Germany). DNA sequencing data were retrieved from the genevestigator.ethz.ch), version of July 2004. Massively parallel signature sequencing data were retrieved from the Arabidopsis massively parallel signature sequencing (MPSS) data base (available on the World Wide Web at mpss.udel.edu/at/java.html), version of August 2004.
Arabidopsis DAG Kinase AtDGK7

(DEF) (Novagen). Cells were grown at 37 °C. At an A600 of 0.6, expression of NuS-A-His-AtDGK7 fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). Cells were harvested 4 h later by centrifugation, resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, and Complete Mini Protease Inhibitor Mixture (Roche Applied Science), and lysed by sonification. Recombinant AtDGK7 was purified using Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer’s protocol. All homogenates were frozen and stored at −80 °C until assayed. Protein concentration was determined according to Bradford (20), using bovine serum albumin as a standard.

Western Blot Analysis—Purified proteins (20 μg) produced by the pET7 plasmid or the pET43c empty vector were separated by SDS-PAGE electrophoresis (21) using a 4% spacer and a 8% (w/v) separating polyacrylamide gel and transferrered onto nitrocellulose membranes (Amersham Biosciences) by semidy electroblotting. For detection, nitrocellulose membranes were blocked overnight in TBS (Tris-buffered saline) containing 0.150 mM NaCl, 0.025 mM Tris/HCl, pH 7, and 5% (w/v) nonfat dry milk (Bio-Rad) prior to a 1-h incubation in anti-His tag antibody (Pierce). After three 15-min washing steps using TBS/TBS plus 0.05% Tween 20 (v/v) to remove residual antibody, membranes were developed with an enhanced chemiluminescence Western blot detection kit (Pierce SuperSignal) and exposed to x-ray films (Kodak X-Omat AR) for 5 s to 5 min.

DGK Enzymatic Assays—Diacylglycerol kinase activity was determined under standard conditions by measuring the incorporation of [γ-32P]ATP into phosphatidic acid at 25 °C as described (18). To assess Michaelis-Menten kinetics, enzymatic activity of DGK was measured as a function of the sum of molar concentrations of CHAPS, Triton X-100, and DAG at a given mol fraction of DAG. Four stock solutions (A, B, C, and D) were prepared. Stock solution A contained 69.5 mM CHAPS, 30 mM Triton, 1 mM DAG (0.01 mM DAG); stock solution B contained 68 mM CHAPS, 30 mM Triton, 2 mM DAG (0.02 mM DAG); stock solution C contained 68 mM CHAPS, 30 mM Triton, 2 mM DAG (0.02 mM DAG); stock solution D contained 65 mM CHAPS, 30 mM Triton, 5 mM DAG (0.05 DAG). Stock solutions were prepared as follows: DAG, dissolved in chloroform/methanol (1:1), was placed in 7-ml Schott glass reaction tubes (square Petri dishes, 100 mm diameter) containing 0.8% agar (Bandelin, Berlin, Germany). Increasing volumes (from 3.6 to 35.0 μl) of the stock solution were pipetted into the glass disposable reaction tubes. The final volume of the reaction mix was 250 μl. Diacylglycerol kinase activity was determined by measuring the incorporation of [γ-32P]ATP into PA as described previously (18). Extraction and separation of phosphorylids were performed as follows: 1 ml of chloroform/methanol (1:1) and 500 μl of 1 M KCl plus 0.2 M H3PO4 were added, and the mixture was mixed thoroughly; samples were centrifuged at 2500 rpm for 5 min in a Labofuge 200 centrifuge (Heraeus Sepatech, Osterode, Germany). The lower phase (lipids) was transferred to a new glass reaction tube and washed once with chloroform/methanol and KCl to discard the remaining radiolabeled ATP. The amount of phosphate incorporated was determined by counting the radioactivity in a liquid scintillation counter.

Enzymatic activity of AtDGK7 was measured as a function of ATP concentration in mixed micelles. A solution containing 50 mM nonlabeled ATP was prepared as stock. Under standard conditions, each enzymatic reaction contained a final concentration of 1 mM nonlabeled ATP and ~5 mCi of [32P]-labeled ATP (18). To assess the effect of varying ATP on the enzymatic reaction, four different ATP final concentrations were tested: 0.10, 1.0, 2.0, and 5.0 mM, respectively. The same amount of radioactive ATP (5 mCi) was added to the assays, and the difference in specific radioactivities obtained were taken into account for calculating the enzyme activities in the individual reactions. For this experiment, the sum of the molar concentrations of CHAPS, Triton X-100, and DAG was 7.20 mM (containing 4.90 mM CHAPS, 2.20 mM Triton, and 0.10 mM DAG).

The effect of the DGK inhibitor R59022 or 3-{2-[4-[[bis-[4-fluorophenyl]methylene]-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone (R59949) on Arabidopsis AtDGK2 and AtDGK7 enzymes was tested at different concentrations of DAG and CHAPS as indicated above. The reaction mix was preincubated for 10 min with DGK inhibitor before the reaction was started by the addition of ATP.

PA standard was obtained from Sigma (product code P 9511). Hartmann Analytic (Braunschweig, Germany) provided [γ-32P]ATP (15 TBq/mmol). Lipids (1,2-SAG, 1,2-DOG, and cardiolipin), salts (MgCl2, LiCl, and NaCl) and detergents (Na-DC and CHAPS) were purchased from Sigma. Serva (Heidelberg, Germany) provided Triton X-100. DAG molecular species 1,2-POG, 1,2-SLG, and 1,2-OPG were purchased from Larodor Fine Chemicals AB (Malmö, Sweden). DGK inhibitors R59022 and R59949 were purchased from Calbiochem.

HPLC Analysis of the Reaction Products of DGK Assay—The products of diacylglycerol kinase assays were obtained by two-phase partitioning. The organic phase was reduced to dryness under a stream of nitrogen gas, and the glycerolipids were deacylated (22). An aliquot of the water-soluble products of deacylation was mixed with l-[U-14C]glycerol 3-phosphate (Amersham Biosciences; specific activity >100 mCi/ mmol) and resolved by anion exchange high performance liquid chromatography with a gradient of (NH4)2HPO4 (23). The column eluate was collected in 1-ml fractions, and 4 ml of Escoinst A scintillation fluid (National Diagnostics, Atlanta, GA) was added. Radioactivity was estimated by dual label scintillation counting in a Wallac 1409 DSA (Turku, Finland) scintillation counter.

Effect of R59022 on Plant Growth and Development—A. thaliana (L.) Heynh. seeds (ecotype C24) were surface-sterilized with 70% ethanol for 5 min and sodium hypochlorite solution (5.0% NaClO, plus 0.05% TWEEN 20) for 5 min, followed by several washes with sterile water. Subsequently, seeds were sown on MS (Murashige Skoog) plates containing 0.8% agar (square Petri dishes, 100 × 100 × 14 mm; NUNC, Wiesbaden, Germany). The DGK inhibitor R59022 was dissolved in dimethyl formamide. Dimethyl formamide (without R59022) was used as control. The seeds were imbibed at 4 °C in the dark for 1 day. Plates were then placed vertically in a growth chamber under long day conditions (16 h of light, 8 h of dark). Lengths of primary roots from the shoot/root transition zone to the root tip were measured. To analyze the effects of the inhibitor R59022 on plant growth and development, 35–100 μM R59022 was added to MS medium containing 0.8% agar.

RESULTS

AtDGK7 cDNA and the Deduced Primary Structure of Its Encoded Protein—In the Arabidopsis genome, seven genes encode putative DGK isoforms that fall into three major clusters. AtDGK2, a member of Cluster I, is a catalytically active enzyme. The AtDGK2 gene is located on chromosome V, and its expression is detected in various tissues of the Arabidopsis plant, including leaves and roots, as previously shown (18).

To investigate whether members of other clusters also encode functional DGKs, we concentrated here on AtDGK7 (encoded by gene locus At4g30340), representing a potential DGK of Cluster II. The AtDGK7 gene is located on chromosome IV, and data base analysis revealed that AtDGK7 is transcribed. In total, four AtDGK7 expressed sequence tags...
GenBank accession numbers AV798976, AV827682, CF773882, and Z26229) and two cDNA sequences (AF360174 and AY113915) obtained through the full-length cDNA cloning projects at RIKEN and SALK can be retrieved from the NCBI database (available on the World Wide Web at www.ncbi.nlm.nih.gov). Because of previous data base annotation ambiguities (reported in Ref. 18), we reanalyzed the AtDGK7 sequence in detail here (see Fig. 1A). RIKEN ESTs AV827682 and AV798976 were derived from clone RAFL 09-18-D16 and represent the 5′/H11032 and 3′/H11032 regions, respectively, of the AF360174 sequence, which in total encompasses 1807 base pairs. The EST Z26229 sequence was obtained from the Versailles EST collection and, like EST AV798976, corresponds to the 3′/H11032 part of the AF360174 full-length cDNA. The EST CF773882 covers the central part of the transcript. Finally, cDNA AY113915, generated at SALK, has a length of 1156 base pairs. The available data can be summarized as follows. (i) The AF360174 cDNA contains a 5′-untranslated region at positions 1–126, the AtDGK7 open reading frame at positions 127–1251, and an unusually long 3′-untranslated region at positions 1252–1807. The presence of this long untranslated region in the AtDGK7 transcript was confirmed by the independently generated EST Z26229. The generation of the long transcript is further in full accordance with data from recent Arabidopsis MPSS analyses (24) (available on the World Wide Web at mpss.udel.edu/at/java.html). A 17-bp MPSS signature sequence corresponding to the very 3′-end of the AtDGK7 gene was found (GATCTATGTTGAGCTTT on the Crick DNA strand). (ii) The nucleotide sequences of the two cDNAs are completely identical in the overlapping region (which encompassed the complete AtDGK7 open reading frame and part of the 3′-untranslated region). Protein sequence analysis reveals that AtDGK7 has a conserved catalytic domain (DGKc; Pfam accession number PF00781) located at amino acid residues 94–240, which contains a presumed ATP-binding site with a GXXGXXG consensus sequence. Although the DGKc of AtDGK7 is ~40% identical to that of AtDGK2, the DGK accessory domain (DGKa; Pfam accession number PF00609) is incomplete in AtDGK7. Furthermore, the two diacylglycerol/ phospholipid binding domains (D/P; InterPro accession number IPR002219) as well as the upstream basic region (UBR) and the extended cysteine-rich domain (extCRD-like domain) are absent from the N-terminal region of AtDGK7. C, amino acid sequence comparison of the DGKc domains of AtDGK2 and AtDGK7. Amino acid residues identical or similar in the two proteins are highlighted in boldface type. The presumed ATP-binding site localized in the DGKc domain (consensus GXXGXXG) is depicted in an open box.
AtDGK7 Transcripts Are Detectable Throughout the Arabidopsis Plant, Especially in Flowers and Seedlings—We could not easily detect AtDGK7 mRNA by standard Northern blot analysis (not shown), indicating weak expression. Therefore, we used real time RT-PCR for the quantitative measurement of AtDGK7 transcripts. To ensure maximum specificity and efficiency during PCR amplification of AtDGK7 cDNA under a standard set of reaction conditions, primer design was done in order to prevent nonspecific amplification of other Arabidopsis genes. Although some Arabidopsis genes have GC-rich promoter regions, AtDGK7 mRNA was shown to contain a guanine-cytosine (GC) content of 55%. The PCR amplicon length was ~100 base pairs. The resulting pair of primer sequences was compared with the Arabidopsis database of expressed sequence tags (ESTs) in the Arabidopsis Information Resource (www.arabidopsis.org) to ensure that AtDGK7 is not transcribed at a low to moderate level, with transcripts per million (TPM) values ranging from 0 to 116. For a weakly expressed gene, this value lies in the range of 1–10 TPM, whereas a very strongly expressed gene may be represented by a value of more than 1000 TPM (for detailed information, see, on the World Wide Web, mpss.udel.edu/at/java.html).

ESTs closely related to AtDGK7 were also reported from other plant species, including Brassica napus (GenBank™ accession number CD826929), Solanum tuberosum (CK256071), and cotton (Gossypium raimondii; CO113886), indicating that AtDGK7-like genes are widely expressed in the plant kingdom.

AtDGK2 Expression Is Rapidly Induced by Wounding—We have previously described the generation of transgenic Arabidopsis plants harboring the promoter region of the AtDGK2 gene (At5g63770) fused to the E. coli β-glucuronidase (GUS) reporter gene (promAtDGK2:GUS reporter lines) (18). Analysis of the promAtDGK2:GUS lines revealed that the AtDGK2 gene is expressed throughout the plant and exhibits cold-inducible gene expression. To search for other environmental factors that could potentially alter AtDGK2 expression, we screened the Genevestigator Arabidopsis microarray data base that provides Affymetrix GeneChip-based transcript profiles from a large number of independent experiments. AtDGK2 transcript level appeared to be slightly induced (~2.3-fold) by nematode attack, as indicated by the Genevestigator Response Viewer tool. Microarray data also indicated that Arabidopsis plants exhibit a strong, but transient, increase of AtDGK2 transcript level in leaves 15 min to 1 h after wounding (viewed with the Digital Northern software package of Genevestigator). At 3 h after wounding, AtDGK2 transcript abundance had already returned back to background level that was observed before induction (Fig. 3A). No changes in gene expression were measured in roots.

In the case of the promAtDGK2:GUS reporter lines, we reproducibly observed strong GUS staining at sites where sections were cut before the incubation in staining solution. Fig. 3B shows an example of wound-induced GUS staining in leaves that had been cut. This result suggested that the activity of the AtDGK2 promoter driving this GUS expression is enhanced upon wounding. Therefore, we quantitatively tested the induction of GUS activity in shoots of promAtDGK2:GUS plants.

To exclude the possibility that the increased GUS activity at the sites of wounding simply results from a better diffusion of substrate into the plant tissue, protein extracts were prepared from control plants and from two representative promAtDGK2:GUS plants harvested at different periods of time after wounding. The two lines were obtained from two independent transformations that employed different binary vectors (pCAMBIA-1303 and pGPTV-HPT, respectively), both carrying the promAtDGK2:GUS reporter lines, we reproducibly observed strong GUS staining at sites where sections were cut before the incubation in staining solution. Fig. 3B shows an example of wound-induced GUS staining in leaves that had been cut. This result suggested that the activity of the AtDGK2 promoter driving this GUS expression is enhanced upon wounding. Therefore, we quantitatively tested the induction of GUS activity in shoots of promAtDGK2:GUS plants.

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To further demonstrate transient, wound-triggered AtDGK2 expression, we performed quantitative real-time RT-PCR experiments, using RNA isolated from 4-week-old plants raised on soil. We observed that perhaps by ozone treatment, as indicated by the Response Viewer tool of Genevestigator.

MPSS data indicate that AtDGK7 is transcribed at a low to moderate level, with transcripts per million (TPM) values ranging from 0 to 116. For a weakly expressed gene, this value lies in the range of 1–10 TPM, whereas a very strongly expressed gene may be represented by a value of more than 1000 TPM (for detailed information, see, on the World Wide Web, mpss.udel.edu/at/java.html).
the AtDGK2 transcript level rose more than 16-fold in two different Arabidopsis accessions tested (i.e. C24 and Col-0) within 30 min after wounding and at later stages went down to the pretreatment level (Fig. 3E). Time course experiments indicated that AtDGK2 expression started to increase as early as 10 min after wounding.

**AtDGK7 Encodes a Functional Lipid Kinase**—We have previously characterized the enzymatic activity of AtDGK2, representing one of the two Cluster I DGK enzymes from *A. thaliana* (18). So far, no enzyme activity has been demonstrated for any member of the Cluster II. We cloned the AtDGK7 cDNA and tested recombinant NusA/polyhistidine-tagged AtDGK7, expressed in *E. coli* (see “Experimental Procedures” for details) for its potential to phosphorylate DAG. After isopropanol-β-D-thiogalactopyranoside induction, protein was extracted for enzymatic analysis. Recombinant fusion protein of an apparent molecular mass of ~100 kDa was detected by an anti-His tag antibody in extracts of the transformed cells (Fig. 4), which compares favorably with the predicted size of the fusion protein (AtDGK7, 41 kDa; NusA tag/His6 tag, ~60 kDa). Extracts from cells transformed with the pET43c control vector (lacking the AtDGK7 cDNA) reacted with the antibody, revealing a protein of ~60 kDa that corresponds to the NusA tag/His6 tag fusion expressed from the empty vector itself.

Proteins purified from cells transformed with the AtDGK7 cDNA or the empty vector (pET43c) were assayed for DGK enzymatic activity. Cells transformed with AtDGK7 showed strong DAG kinase activity against 1,2-DOG and 1,2-SAG, both of which contain unsaturated fatty
Arabidopsis DAG Kinase AtDGK7

FIGURE 5. Enzyme activity of AtDGK7. Purified recombinant AtDGK7 catalyzes the in vitro phosphorylation of DAG to yield PA, using [γ-32P]ATP as phosphate donor, according to the protocol described elsewhere (18). A, products of the enzymatic reaction were solubilized in chloroform/methanol (2:1) and subsequently separated by thin layer chromatography and detected by autoradiography (left panel) and iodine vapor (right panel). The generated radiolabeled PA detected by autoradiography was identified by comparison with standard PA. Retention factor value for PA standard, radiolabeled PA, and cardiolipin is 0.95. Both the left and right panels contain the following. Lane 1 and 2, labeled PA produced by the activity of purified AtDGK7 enzyme, obtained from independent bacterial cultures transformed with the AtDGK7 cDNA (plasmid pET7), using 1,2-DOG (lane 1) or 1,2-SAG (lane 2) as substrate; lane 3, purified NusA-His6 fusion protein expressed from the cloning vector pET43c alone; lane 4, no enzyme added to the reaction mix; lane 5, PA standard; lane 6, cardiolipin. B, diacylglycerol substrate was incubated with affinity-purified AtDGK7 enzyme. The lipid products of assay were deacylated and mixed with [32P]glycerol 3-phosphate, and the water-soluble products of deacylation were resolved on a Partisphere SAX HPLC column; open symbols, 14C; closed symbols, 32P. The position of elution of the internal standard is indicated with an arrow, as is the position of elution of an internal standard of AMP, monitored on-line at 258 nm.

acid moieties, whereas the transformants harboring the empty vector exhibited no activity after autoradiography of TLC plates (Fig. 5A), demonstrating that AtDGK7 encodes a functional lipid kinase. This result was confirmed by HPLC analysis (Fig. 5B). Deacylation and subsequent HPLC of the 32P-labeled products obtained by incubation of enzyme with 1,2-DOG yielded a 32P-labeled peak that co-eluted with an internal standard of authentic 14C-glycerol 3-phosphate.

Using standard experimental conditions (described in Ref. 18), incorporation of radioactive label was linear with time for at least 30 min in the activity assay. Therefore, in order to measure DGK activity, PA formation was determined in this linear kinetic range. Enzyme assays of purified recombinant AtDGK7 showed that the reaction was linear for up to ~2 µg of protein in the assay, using 1,2-DOG as substrate (Fig. 6A). Therefore, 1 µg of protein was used in all further experiments.

The AtDGK7 enzyme has an activity optimum at pH 6.8 and exhibits a fairly rapid decrease of activity below pH 6.8 and above pH 7.8 (Fig. 6B). NaCl (5–50 mM) increased the activity of the enzyme by up to 20% as compared with the standard assay. In the presence of LiCl, the highest enzyme activity was observed at a concentration of 50 mM. When the concentration of both salts was raised to 500 mM, the activity of the protein began to decrease (Fig. 6C). AtDGK7 activity was tested in the presence of different types of detergents. Sodium deoxycholate (Na-DC) at 1–5 mM concentration proved to be an efficient detergent for solubilization of lipids. Enzyme activity strongly decreased at higher Na-DC concentrations (10–100 mM). In contrast, Triton X-100 (0.07–0.52%) only marginally affected AtDGK7 activity in the presence of 1 mM Na-DC (Fig. 6D). Low concentrations of CHAPS (1–5 mM) replacing Na-DC were inefficient to activate the enzyme, whereas higher concentrations of this detergent (10–50 mM) strongly improved AtDGK7 activity. An inhibitory effect was observed at 100 mM CHAPS. At 1 mM CHAPS, 0.12–0.2% Triton X-100 strongly increased (around 8-fold) AtDGK7 activity. At the highest Triton X-100 concentration tested (2.02%), enzyme activity exceeded by almost 40% the activity determined under control conditions (Fig. 6E).

Because both the substrate and product of the DGK reaction are amphiphilic and not freely diffusible in aqueous solution, simple Michaelis-Menten kinetics might not be suitable to describe the enzymatic characteristics of this enzyme. The surface dilution kinetic model was developed for enzymatic reactions occurring at surfaces of mixed micelles in aqueous environment (27). This model is based on an enzyme converting amphiphilic substrates into reaction products in detergent-containing mixed micelles. Because the reaction partners localize to the surface of the micelles, they can only diffuse in two dimensions, and different kinetics apply in contrast to normal Michaelis-Menten kinetics, which are based on three-dimensional diffusion of enzymes, substrates, and products.

The surface dilution model predicts that enzyme activity should depend on the molar and micellar surface concentrations of the substrate when the molar substrate concentration relative to the other micellar components is low but be predominantly dependent on the surface concentration and be independent of the molar substrate concentration when the molar substrate concentration relative to detergent is high (27, 28). To test whether the surface dilution model is suitable to describe DGK kinetics, we measured DGK activity as a function of the total molar concentration of the three amphiphiles: CHAPS, Triton X-100, and diacylglycerol. CHAPS and Triton X-100 were considered as a single complex detergent. Fig. 7 demonstrates that within a range of total ([CHAPS] + [Triton X-100] + [diacylglycerol]) concentration between 5 and 8 mM, DGK activity was largely independent of the diacylglycerol molar concentration but dependent on the diacylglycerol surface concentration (given in mol fraction of diacylglycerol relative to total amphiphiles).

This result demonstrates that under our experimental conditions, DGK activity of both enzymes follows the surface dilution kinetic model. Fig. 7 shows that the specific activities for AtDGK2 and AtDGK7 were in the range of 0.5–3 pmol of PA min−1 µg−1 protein. Biochemical studies characterizing wheat (14) and tomato (15) DGK isoforms have reported specific activity values of up to 20 pmol of PA min−1 µg−1 protein, which are significantly higher than that observed here for the Arabidopsis enzymes.

Kinetic data for AtDGK2 and AtDGK7 were obtained from Michaelis-Menten kinetics using the surface dilution model. Calculated \( K_m \) and \( V_{max} \) values were obtained for 1,2-DOG using an ATP concentration of 1 mM and different total amphiphile concentrations of 1.44, 7.2, or 14 mM (Fig. 8, A and B). The \( K_m \) values for AtDGK2 and AtDGK7 at the three different total amphiphile concentrations were very similar, around 0.1 mol fractions of 1,2-DOG. The \( V_{max} \) values were in the same range for AtDGK2 and AtDGK7 but were dependent on the total amphiphile concentration (Fig. 8). This means that with increasing amounts of the total ([CHAPS] + [Triton X-100] + [diacylglycerol])
mixture, the $V_{\text{max}}$ for AtDGK2 and AtDGK7 decreased. This can be explained by the surface dilution model, because by adding more and more detergent (and substrate), new and larger micelles are formed, whereas the amount of the enzyme remains the same. Enzyme is diluted out, because the reaction volume (which in this case is only two-dimensional) is enlarged. The inhibition of AtDGK2 activity by high concentrations of CHAPS was already found by Gómez-Merino et al. (18). Furthermore, $V_{\text{max}}$ measured for AtDGK2 in the presence of CHAPS
was much higher than previously reported in experiments done in the absence of CHAPS (0.76 pmol of PA min\(^{-1}\) g\(^{-1}\); see Ref. 18), indicating that low amounts of CHAPS stimulate AtDGK2 activity.

The effect of increasing concentrations of ATP on the enzymatic activities of AtDGK2 and AtDGK7 was determined using a sum of the molar concentrations of CHAPS, Triton X-100, and DAG of 7.2 mM at 0.02 mol fractions of 1,2-DOG (Fig. 9). AtDGK7 activity was compared with that of AtDGK2 under these experimental conditions. We observed that AtDGK2 was about 3.5-fold more active than AtDGK7, as its \(V_{\text{max}}\) reached 1.20 pmol of PA min\(^{-1}\) g\(^{-1}\) protein, as compared with 0.34 pmol PA min\(^{-1}\) g\(^{-1}\) protein for AtDGK7. The \(V_{\text{max}}\) values obtained in this experiment were lower than in the assays done with different amounts of 1,2-DOG (see above), indicating that the amount of 1,2-DOG used in this experiment (0.02 mol fractions) was not saturating. For AtDGK2 the \(K_{\text{m}}\) for ATP was 1.20 mM. The \(K_{\text{m}}\) of 0.74 mM for ATP in the case of AtDGK7 is somewhat lower and might indicate the high affinity of this enzyme for this substrate.

**R59022 Differentially Affects Enzymatic Activity of AtDGK2 and AtDGK7 and Alters Root and Plant Growth**—The DGK inhibitor R59022 modulates a number of cellular processes in animals that are regulated by DAG/PA signaling (e.g. see Refs. 29 –31). In plants, R59022 has been demonstrated to reduce the formation of cold-induced PA in A. thaliana suspension cells at a concentration of around 50 –100 \(\mu\)M of the inhibitor (32). It also enhanced phytoalexin accumulation in elicitor-treated (but not untreated) epicotyl tissues of pea, most likely involving an activation of phenylalanine ammonia lyase gene expression (33). Membrane-bound DGK activity in pea was inhibited 50% at an inhibitor concentration of around 100 \(\mu\)M (33). We have previously reported that R59022 also reduces the activity of recombinant AtDGK2 at relatively low concentrations (IC\(_{50}\) 50 \(\mu\)M) (18). To investigate whether AtDGK7 is inhibited in a similar manner, the reaction mix was preincubated with this inhibitor. Since the standard conditions for these experiments were different from those previously reported (dimethylformamide used as a solvent for R59022, instead of dimethyl sulfoxide) (18) and three different molar fractions were tested herein, we assayed both AtDGK2 and AtDGK7. Our data indicate that the decrease in PA formation and, hence, the degree of inhibition of DGK activity were indeed dependent on the concentration of the inhibitor (Fig. 10). R59022 had a stronger inhibitory effect on AtDGK2 (IC\(_{50}\) = 250 \(\mu\)M) in comparison with AtDGK7 (IC\(_{50}\) = 500 \(\mu\)M). Notably, at 50 \(\mu\)M R59022, no inhibition was detected for AtDGK7, whereas AtDGK2 was inhibited by 20%. At the highest R59022 concentration tested (1000 \(\mu\)M), AtDGK2 was still 20% active, whereas AtDGK7 was ~30% active. Additionally, we tested the effect of a second DGK inhibitor, R59949 (up to 1 \(\mu\)M in the reaction mix), but no inhibitory effect was observed, neither for AtDGK2 nor for AtDGK7 (data not shown). In animals, R59949
inhibits Ca\(^{2+}\)-activated DGKs, but Ca\(^{2+}\)-insensitive isoforms are only weakly affected (34). We have previously reported that AtDGK2 activity is not modified by Ca\(^{2+}\) in the reaction assay (18).

Evidence indicates that DGKs may regulate diverse cellular processes such as growth and differentiation in animals (10). In plants, studies involving the DGK inhibitor R59022 demonstrated an involvement of DGK in stress-related physiological processes (cold, elicitor treatment) (32, 33). We have shown that AtDGK2 is prominently expressed under non-stress conditions in root tips of seedlings a few days after germination (18), indicating that it plays a developmental role in this tissue. Only weak expression in roots was observed for AtDGK7 (this report; Fig. 2A). We grew plants in the presence of the DGK inhibitor R59022 to assess whether it affects root development. To this end, Arabidopsis seedlings were grown on agar plates (kept vertically) in the absence and presence of the inhibitor. Primary root elongation rate was analyzed and found to be strongly reduced at an inhibitor concentration above 50 \(\mu\)M (Fig. 11, A and B). At a concentration of 75 \(\mu\)M R59022, root elongation was inhibited by \~\(50\%\). R59022 also inhibited lateral root growth (not shown).

The experiments reported in this paper were initiated as an attempt to demonstrate that a member of Cluster II of plant DGKs is a functional enzyme, since no protein of such a cluster has been characterized so far from any species. AtDGK7 and previously characterized AtDGK2 share a well conserved catalytic domain, although the primary structure of the former is far simpler than the latter. The complex domain organization present at the N-terminal region of AtDGK2, which contains two copies of a DAG/PE-binding domain flanked by an upstream basic region preceding the DAG/PE-binding domain 1, and an extCRD-like sequence following the DAG/PE-binding domain 2, is absent in AtDGK7 (Fig. 1B). Furthermore, the DGK accessory domain is incomplete in AtDGK7.

In contrast to AtDGK2, AtDGK7 transcripts were not detected under conventional Northern blot conditions. Quantitative RT-PCR revealed that AtDGK7 is expressed throughout the plant, most prominently in flowers and seedlings. Affymetrix GeneChip hybridization studies so far have not revealed any profound affect of biotic or abiotic stresses on AtDGK7 transcript levels, suggesting that AtDGK7 may be more involved in regulating developmental or growth processes than in stress responses. In contrast, we have previously reported that expression of AtDGK2 is induced by cold treatment, indicating an involvement in cold-related signal transduction (18). Here, we observed that wounding also triggers AtDGK2 expression. Wounding is known to rapidly trigger the accumulation of PA in plants (36). Molecular-physiological evidence indicates that this rise in PA level to a large extent results from an involvement of phospholipase D activity. However, other biochemical pathways contributing to PA production under stress, such as cold (18, 32) and wounding (36), are likely. Our finding that AtDGK2 expression rapidly reacts to a wound stress is consistent with this interpretation and conforms to the model that genes that code for signal pathway components are themselves often triggered by the stimulus that is transmitted by the signaling pathway (37).

The AtDGK7 gene encodes a functional diacylglycerol kinase with unique biochemical properties. AtDGK7 saturates at lower substrate concentrations, and its activity appears to be approximately 3–4 times

**DISCUSSION**

AtDGK2 and AtDGK7 Phosphorylate 1,2-DAG Molecular Species Physiologically Important in Plants—Higher plants contain a variety of diacylglycerol molecular species that differ by the length and degree of unsaturation of the acyl groups linked in the sn-1- and sn-2-position of the glycerol backbone. Two major sites contribute to diacylglycerol formation in many plants (e.g. Arabidopsis) (i.e. the chloroplast and the ER). Due to differences in the specificity of the acyltransferases involved, two major types of diacylglycerol can be distinguished: a prokaryotic type derived from the plastid (C18 at sn-1, mostly C16 at sn-2), or a eukaryotic type (C16 or C18 at sn-1, C18 at sn-2) (35). We previously have proved that AtDGK2 has high specificity for 1,2-SAG and 1,2-DAG (18). To further investigate the ability of AtDGK2 and AtDGK7 to phosphorylate 1,2-sn-diacylglycerides found in plants, we tested 1,2-POG/1-palmitoyl, 2-oleoyl-sn-glycerol (1–16:0–2–18:1); 1,2-SLG/1-stearoyl, 2-linoleoyl-sn-glycerol (1–18:0–2–18:2); and 1,2-OPG/1,2–1-oleoyl, 2-palmitoyl–sn-glycerol (1–18:1–2–16:0) (Fig. 1). Interestingly, AtDGK2 activity increases by 46% with 1,2-SLG as substrate in comparison with the control (1,2-DAG). With 1,2-POG and 1,2-OPG used as substrates, AtDGK2 activity is more than 30% higher than the control. Regarding AtDGK7, our data indicate that this enzyme exhibits elevated activity in the presence of 1,2-POG and 1,2-OPG (nearly 20% higher than with 1,2-DAG). With 1,2-SLG, AtDGK7 activity is similar to that observed with the control substrate.

**FIGURE 10. Effect of the DGK inhibitor R59022 on AtDGK2 and AtDGK7 activities using different lipid mol fractions.** The DGK inhibitor R59022, dissolved in dimethyl formamide (DMF), was added prior to the start of the reaction containing AtDGK2 (upper panel) or AtDGK7 (lower panel) protein. Enzymatic activity was determined as described under “Experimental Procedures.” Values are expressed as percentages of enzyme activity determined under control assay conditions (i.e. in the absence of R59022) showing the highest activity. Measurements were done as a function of the sum of molar concentrations of CHAPS, Triton X-100, and DAG at a given mol fraction. A, 1.44 \(\mu\)M (1.00 \(\mu\)M CHAPS, 0.43 \(\mu\)M Triton, 0.01 \(\mu\)M DAG); B, 3.60 \(\mu\)M (2.50 \(\mu\)M CHAPS, 1.08 \(\mu\)M Triton, 0.02 \(\mu\)M DAG); C, 7.20 \(\mu\)M (4.90 \(\mu\)M CHAPS, 2.20 \(\mu\)M Triton, 0.10 \(\mu\)M DAG). Data are means of three independent determinations \pm S.D.
lower than that of AtDGK2, possibly due to its partial DGK accessory domain. AtDGK7 is the smallest of the plant DGKs known (containing 374 amino acids) and apparently is also smaller than all other known eukaryotic DGKs described so far. Because of this, we can assume that all other DGKs encoded by the Arabidopsis genome are bona fide diacylglycerol kinases. Although an Arabidopsis Cluster III enzyme has not been analyzed biochemically so far, two isoforms of a Cluster III enzyme from tomato have been shown to be catalytically active (15).

AtDGK2 and AtDGK7 are differentially affected by pH, detergents, and the inhibitor R59022. The optimal pH found at 6.8 for recombinant AtDGK2 is 0.4 pH units lower than that of AtDGK2 (i.e. 7.2). Inhibitory effects of salts (800 mM NaCl) have been observed in a DGK isoenzyme from Rattus norvegicus (38). Interestingly, at concentrations below 500 mM LiCl and NaCl positively affected AtDGK7 activity. Instead, AtDGK2 activity is reduced when those salts are used at concentrations above 200 mM in the reaction mix. AtDGK7 activity is dependent on the presence of detergents. Increasing concentrations of the anionic detergent Na-DC decrease PA formation, whereas the nonionic detergent Triton X-100 does not. An opposite effect is observed with CHAPS, since enzymatic activity of AtDGK7 in buffers containing such zwitterionic detergent at low concentrations (i.e. 5 and 10 mM) is reduced.

Diaclylglycerol found in plant membranes can be derived from PA phosphatase action on PA originating from de novo synthesis in the ER or the plastid. However, alternative pathways exist for diacylglycerol production from membrane lipids via phospholipase C or phospholipase D/PA phosphatase reaction. Therefore, the molecular species distribution of diacylglycerol is complex and depends on the subcellular membrane analyzed. Not much is known about the molecular species specificity of enzymes involved in diacylglycerol metabolism, and its analysis is hampered by the fact that only a few of the diacylglycerol molecular species presumed to be important in plant lipid metabolism are commercially available. We therefore focused our analysis on the specificity for diacylglycerols having a eukaryotic (1,2-DOG, 1,2-POG) or prokaryotic (1,2-OPG) structure or diacylglycerols with different degrees of unsaturation (1,2-SLG). Both enzymes, AtDGK2 and AtDGK7, were about 20–30% more active with 1,2-POG and 1,2-OPG as compared with 1,2-DOG. Therefore, diacylglycerol molecular species containing at least one saturated fatty acid seem to be the preferred substrates for both enzymes. In accordance with this finding, AtDGK2 showed higher activity with 1,2-SAG (18), and 1,2-SLG (this report), two substrates carrying stearate at the sn-1-position. However, no increase in AtDGK7 enzyme activity was observed when comparing 1,2-DOG (two monounsaturated acyl groups) and 1,2-SLG (stearate in sn-1, diunsaturated fatty acid in sn-2). AtDGK2 and AtDGK7 display different specificities for diacylglycerol substrates of different subcellular origin (eukaryotic: 1,2-POG, 1,2-DOG; prokaryotic: 1,2-OPG).
However, the significance of this finding is unclear, because the subcellular location for none of the respective enzymes, AtDGK2 or AtDGK7, is currently known.

The DGK inhibitor R59022 differentially affects the enzymatic activity of AtDGK2 and AtDGK7. In AtDGK7 enzymatic assays, half-maximal inhibition (IC$_{50}$) is reached at an R59022 concentration almost 2-fold higher than that observed for AtDGK2, which possibly could be explained in light of the complex domain organization of AtDGK7 that may result in a higher affinity of the inhibitor for the enzyme. Recently, R59022 has been shown to minimize cold-induced PA formation in _A. thaliana_ suspension cells (32) and to stimulate phytoalexin accumulation in elicitor-treated pea epicotyl tissues (33), indicating an involvement of DGK in both stress-related physiological responses. In the present report, we further demonstrate that R59022 also affects growth and development. Root elongation was drastically reduced in the presence of 50–100 μM R59022, a concentration range that was found to partially inhibit recombinant AtDGK2 in _vitro_. We therefore suggest that DGKs play an important role not only in stress responses but also in developmental processes in plants, consistent with the observation that _AtDGK2_ transcriptional activity undergoes a developmental shift during root growth (18). Although we have demonstrated here that R59022 inhibits plant DGK activity _in vitro_, the possibility cannot be excluded at present that other cellular proteins interact with the inhibitor, thereby triggering the developmental and physiological effects that we and others have observed.

In animals, DGKs play a pivotal role in many biological processes, such as cell proliferation, differentiation, survival, and apoptosis (10). Nuclear DGKs are associated with other regulatory enzymes of the phosphoinositide cycle and have an effect on cell cycle progression (39). In animals, DGKs play a pivotal role in many biological processes, such as cell proliferation, differentiation, survival, and apoptosis (10). Nuclear DGKs are associated with other regulatory enzymes of the phosphoinositide cycle and have an effect on cell cycle progression (39).

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AtDGK7, the Smallest Member of Plant Diacylglycerol Kinases (DGKs), Displays Unique Biochemical Features and Saturates at Low Substrate Concentration: THE DGK INHIBITOR R59022 DIFFERENTIALLY AFFECTS AtDGK2 AND AtDGK7 ACTIVITY IN VITRO AND ALTERS PLANT GROWTH AND DEVELOPMENT

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