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Rapid phosphatidic acid accumulation in response to low temperature stress in *Arabidopsis* is generated through diacylglycerol kinase

*Steven A. Arisz¹, Ringo van Wijk¹, Wendy Roels¹, Jian-Kang Zhu²,³, Michel A. Haring¹ and Teun Munnik*¹*

¹ Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands  
² Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, USA  
³ Shanghai Center for Plant Stress Biology and Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China

**INTRODUCTION**

The potential to survive low temperatures is one of the factors that determine the geographical distribution of plants. Moreover, freezing and cold stress restrict the arable land and yield of crops. Therefore, much effort is made to understand the mechanisms that make plants more tolerant to low temperatures. One of the most popular plant models in these studies is *Arabidopsis thaliana* (Van Buskirk and Thomashow, 2006).

Like many temperate plants, *Arabidopsis* is capable of cold acclimation, i.e., during a period of cold, non-freezing temperatures, its tolerance for freezing temperatures increases. This process, also referred to as cold hardening, involves a myriad of metabolic and developmental changes, accompanied by accumulation of proteins and compatible solutes, and alterations in membrane composition (Uemura et al., 1995; Thomashow, 1999; Cook et al., 2004; Chinnusamy et al., 2007).

Transcriptome profiling and mutant screens have resulted in the characterization of multiple genes involved in the initiation of cold acclimation and freezing tolerance. These include the conserved CBF/DREB1 transcription factors that are responsible for activating the expression of many cold response (COR) genes via conserved C-repeat elements in their promoters. Zhu and coworkers have used *Arabidopsis* plants transfected with the *RD29A-LUC* construct to select for mutants with altered responses to cold treatment ("cold response mutants"), resulting in the identification of several genes. Enhanced cold-induced expression was found in the *fry1* (Xiong et al., 2001) and *hos1* (Ishitani et al., 1998) mutants, whereas *los1* (Guo et al., 2002) showed decreased expression. Moreover, the dominant negative *ice1* (Chinnusamy et al., 2003) mutation has been demonstrated to negatively affect cold-induced gene transcription by interfering with the function of AtICE1, a *myc*-type transcription factor which functions in CBF transcription in cold signaling. The *myb*-type transcription factor SNOW1/MBY15, also binds to the CBF promoter region, interacting with ICE1 (Agarwal et al., 2006). Upon exposure to cold stress (4°C), the transcript levels of CBF/DREB1 genes increase within 15–30 min, followed by the accumulation of COR gene transcripts after about 2 h (Thomashow, 1999).

Phosphatidic acid (PtdOH) is emerging as an important signaling lipid in abiotic stress responses in plants. The effect of cold stress was monitored using ³²P-labeled seedlings and leaf discs of *Arabidopsis thaliana*. Low, non-freezing temperatures were found to trigger a very rapid ³²P-PtdOH increase, peaking within 2 and 5 min, respectively. In principle, PtdOH can be generated through three different pathways, i.e., (1) via de novo phospholipid biosynthesis (through acylation of lyso-PtdOH), (2) via phosphorylation of diacylglycerol (DAG) by DAG kinase (DGK). Using a differential ³²P-labeling protocol and a PLD-transphosphatidylation assay, evidence is provided that the rapid ³²P-PtdOH response was primarily generated through DGK. A simultaneous decrease in the levels of ³²P-PtdInsP, correlating in time, temperature dependency, and magnitude with the increase in ³²P-PtdOH, suggested that a PtdInsP-hydrolyzing PLC generated the DAG in this reaction. Testing T-DNA insertion lines available for the seven DGK genes, revealed no clear changes in ³²P-PtdOH responses, suggesting functional redundancy. Similarly, known cold-stress mutants were analyzed to investigate whether the PtdOH response acted downstream of the respective gene products. The *hos1, los1*, and *fry1* mutants were found to exhibit normal PtdOH responses. Slight changes were found for *ice1, snow1*, and the overexpression line *Super-ICE1*, however, this was not cold-specific and likely due to pleiotropic effects. A tentative model illustrating direct cold effects on phospholipid metabolism is presented.

**Keywords:** abiotic stress, cold stress, diacylglycerol kinase, lipid signaling, phosphatidic acid, phosphoinositide, phospholipase, plant signaling
Much less is known about the signal transduction pathway that precedes the gene expression changes. Nonetheless, there is mounting evidence that Ca$^{2+}$ functions as a second messenger (Knight et al., 1996; Knight and Knight, 2000; Carpaneto et al., 2007) and that part of the pathway involves activation of a MAP kinase cascade (Jonak et al., 1996; Mizoguchi et al., 1996; Teige et al., 2004). One of the latest additions to the field of cold signaling is the formation of the lipid second messenger, phosphatidic acid (PtdOH). In suspension-cultured cells, this phospholipid was shown to accumulate within minutes of cold stress (Gawer et al., 1999; Ruelland et al., 2002; Cantrel et al., 2011). Like Ca$^{2+}$ and MAP kinases, PtdOH is involved in the signal transduction pathways of several other plant stress responses, including drought, wounding, and pathogen infection (Li et al., 2009; Testerink and Munnik, 2011), and it is not unlikely that these pathways strongly overlap.

In stress-induced signal transduction, PtdOH responses have been mainly attributed to two pathways. It is the direct product of phospholipase D (PLD), which hydrolyses structural phospholipids like phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtN), and a secondary product of the phospholipase C (PLC) pathway, which first hydrolyzes polyphosphoinositides (PPIs) to diacylglycerol (DAG), that is subsequently phosphorylated to PtdOH by diacylglycerol kinase (DGK). However, metabolism of DAG and PtdOH is more complex, since multiple sources have now been demonstrated, and PtdOH is formed de novo via acylation of glycerolipid (Gro3P) as a common intermediate in glycerolipid biosynthesis, both in the plastid and the ER. Thus, PtdOH is precursor to all phospholipids as well as triacylglycerols and galactolipids, and its turnover is crucial in determining lipid metabolic fluxes and membrane compositions.

The *Arabidopsis* genome is predicted to encode 12 PLDs, 9 PLCs, and 7 DGKs (Gomez-Merino et al., 2004; Testerink and Munnik, 2005, 2011; Tasma et al., 2008; Arisz et al., 2009; Li et al., 2009; Munnik and Testerink, 2009). Their genetic abundance and specific gene expression patterns suggest that some of these enzymes are specific to certain locations in specific organs and/or involved in distinct processes. The PLC/DGK and PLD pathways have been implicated in the transciptional induction of an array of cold-induced genes in *Arabidopsis* (Vergnolle et al., 2005). PLDδ has been shown to be important in the generation of freezing tolerance during acclimation (Li et al., 2004) in contrast to PLDα1 which negatively influenced survival of freezing, both in cold-acclimated and in non-acclimated plants (Rajashekar et al., 2006; Chen et al., 2008; Du et al., 2010). In suspension-cultured cells, biochemical evidence was found that cold shock activated both PLC/DGK and PLD pathways (Rueland et al., 2002). Moreover, several genes have been shown to be upregulated in response to cold stress, including PLDα1, PLDβ, PLC1, PLC4, PLC5, DGK1, and DGK2 (Hirayama et al., 1995; Gomez-Merino et al., 2004; Li et al., 2004; Lee et al., 2005). Also, in maize roots and leaves several genes encoding DGK, PLC, and PLD were upregulated within 30 min of cold stress (Sui et al., 2008).

In this study we show that *Arabidopsis* seedlings and leaf disks exposed to low temperatures accumulate PtdOH within minutes. Using a differential 32P-labeling strategy (Munnik et al., 1998b; Arisz et al., 2009) and PLD’s ability to transphosphatidylate n-butanol to PtdBut (Munnik et al., 1995, 1998b), we provide evidence that the rapid PtdOH response does not originate from PLD but from DGK. The simultaneous decrease in the level of phosphatidinositolphosphosphate (PtdInsP) suggests the involvement of a PtdInsP-hydrolyzing PLC. T-DNA insertion lines were used to address the question which DGK and PLC were involved, while the COR mutants hos1, los1, fry1, ice1, and snow1 were analyzed to see whether PtdOH acts up- or down-stream of these genes in the COR.

**MATERIALS AND METHODS**

**PLANT MATERIAL**

*A. thaliana* seeds were sterilized in 70% EtOH (1 min) and 25% bleach (20 min), and sown on media in Petri dishes. For 32P-radiolabeling experiments, seedlings were grown on ½ x Murashige and Skoog (MS) basal medium at pH 5.7 (KOH), solidified with 1% sucrose. A 16 h light/8 h dark regime (150 umol m$^{-2}$s$^{-1}$) at 21°C was set. To promote uniform germination, plates were kept in the dark at 4°C for 2 days before transfer to a climate room.

**RT-PCR EXPRESSION ANALYSES OF DGK T-DNA INSERTION LINES**

Homozygous T-DNA insertion lines of the DGK genes were genotyped using primer sequences found in Table A1 (Figure A3). Wild type *A. thaliana Col-0* or lines containing T-DNA insertions in *DGK1, -3, -5, -7* genes were grown on ½ x Murashige and Skoog (MS) basal medium supplemented with 1% w/v sucrose at pH 4.6, solidified with 1% w/v daishin agar. To promote uniform germination, plates were kept in the dark at 4°C for 2 days before transfer to a climate room. Seedlings were collected from plants grown in a greenhouse under the same environmental conditions. RNA was isolated using Tri Reagens LS (Sigma) and treated with Turbo RNAse free DNAse (Ambion) for removal of genomic DNA. The RNA concentration and integrity was analyzed using a Nanodrop ND-1000 spectrophotometer.

cDNA was synthesized from 2 μg total RNA using RevertAid H Minus Reverse Transcriptase (Fermentas) according to the manufacturers protocol. RT-PCR was performed using Accuprime Taq DNA polymerase (Invitrogen). Table A2 contains the primer sequences used to amplify the different *Arabidopsis* DGK genes and the At2g28390 (SAND family) reference gene (Figure A4; Hong et al., 2010). Thermal cycling was done according to the following profile: 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 2 min and 1 cycle of 68°C for 6 min.

**32P-ORTHOPHOSPHATE RADIOLABEling in vivo AND ANALYSIS OF PHOSPHOLIPIDS**

Five-days-old seedlings or leaf disks (5 mm Ø) of 3-weeks-old plants were transferred to a 2.0 ml Eppendorf tube, containing...
MES (2-[N-morpholino]ethane sulfonic acid)-based buffer of 2.56 mM MES (pH 5.7) and 1 mM KCl. To label phospholipids, 10 μCi carrier-free 32P-orthophosphate per tube was added for 16 h, unless indicated otherwise. Cold shock treatments were executed by transferring tubes to ice water. Incubations were stopped by the addition of HClO4 (final concentration 5%, w/v), and 10 min of subsequent shaking.

The total solvent was removed and 375 μl CHCl3/MeOH/HCl (50:100:1, by vol.) was added to extract the lipids. After 10 min of vigorous shaking, two phases were induced by adding 375 μl CHCl3 and 200 μl 0.9% (w/v) NaCl. The organic lower phase was then transferred to a tube containing 375 μl CHCl3/MeOH/1M HCl (3:48:47, by vol.). Shaking, spinning, and removing the upper phase yielded a purified organic phase, which was dried down in a vacuum centrifuge at 50°C. The residue was resuspended in 50 μl CHCl3 and sampled for lipid analysis.

Phospholipids were analyzed by thin-layer chromatography (TLC) on heat-activated silica gel 60 plates (Merck, 20 × 20 cm) using one of the following solvent systems (ratios by vol.): (A) CHCl3/MeOH/NH4OH (25%)/H2O (90:70:4:16); or (B) ethylacetate/iso-octane/formic acid/H2O (13:2:3:10), of which the organic phase was used for TLC. Solvent A was used for total phospholipid analysis, while B was used to quantitate PtdOH and PtdBut. Radiolabeled phospholipids were visualized and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale CA, USA).

RESULTS
COLD STRESS RAPIDLY TRIGGERS A PtdOH RESPONSE
PtdOH levels in plants are approximately 2 mol% of total phospholipids (Welti et al., 2002) which likely represents ER- and plastid-localized PtdOH as precursor and turnover product of structural glycerolipids. To be able to see PtdOH increases during stress-signaling, plants can be metabolically radiolabeled with carrier-free 32P-phosphate (32P). To study phospholipid metabolism during cold shock in Arabidopsis, we radiolabeled 5-days-old seedlings for 16 h with 32P and subsequently incubated them for 5 min at 0°C. Phospholipids were then extracted, separated by TLC and analyzed by autoradiography. A typical 32P-labeling pattern is shown in Figure 1, revealing a PtdOH increase in response to cold. To test the temperature dependency of this response, 32P-prelabeled seedlings were exposed to different temperatures for 5 min. As shown in Figures 2A,B, a temperature-dependent PtdOH response was found. Concomitantly, a decrease in 32P-PtdInsP was observed (Figure 2A). To investigate whether leaves of adult plants responded similarly, leaf disks of 3-weeks-old plants were subjected to the same labeling procedure whether leaves of adult plants responded similarly, leaf disks of 3-weeks-old plants were subjected to the same labeling procedure.

The kinetics of the PtdOH response was investigated. As shown in Figure 3A, PtdOH accumulation at 0°C in seedlings reached a maximum within 2 min and then leveled off, staying up for at least 2 h. The response of leaf disks of adult plants to 0°C was found to be slightly slower but was still relatively fast, peaking at 5 min after the onset of incubation after which it leveled off, approaching control levels after 2 h (Figure 3B).

THE RAPID COLD INDUCED-PtdOH RESPONSE IS GENERATED BY DGK, NOT BY PLD ACTIVITY
Next, we focused on the metabolic origin of the cold-induced PtdOH response. Previous studies in suspension-cultured Arabidopsis cells indicated that part of the cold shock-induced PtdOH response was generated by PLD activity (Ruelland et al., 2002). To investigate PLD’s contribution a transphosphatidylation-assay was performed, i.e., in the presence of a low concentration of a primary alcohol, such as n-ButOH, this serves as a substrate in a PLD-catalyzed reaction generating PtdBut, at the cost of PLD-catalyzed production of PtdOH (Munnik et al., 1995). The accumulation of PtdBut is a measure of PLD activity. Thus, seedlings were prelabeled for 16 h with 32P, then n-ButOH (0.5% final conc.) was added, and 30 min later the seedlings were transferred to 0°C for 5 min or kept at room temperature. As shown in Figure 4A, cold stress did not affect 32P-PtdBut levels, while 32P-PtdOH levels increased. These data indicate that PLD is not responsible for the initial PtdOH response.

To investigate the potential involvement of DGK, a differential radiolabeling protocol was applied (Munnik, 2001; Arisz et al., 2009). In short, when cells are metabolically labeled with 32P1, the phospholipid classes are labeled with different kinetics, depending on the labeling of their precursors, their rates of synthesis, turnover, and pool size. Thus, DGK-derived PtdOH is labeled after relatively short labeling times because it acquires its 32P-phosphate directly from ATP molecules, which are rapidly labeled. This is in contrast to PtdOH arising from PLD activity, which are not labeled until the pool of its precursor, i.e., PtdEtn,
PtdCho, or PtdGro, is sufficiently labeled, which is typically O/N (Munnik et al., 1998b; Arisz and Munnik, 2011).

Thus, seedlings were $^{32}$P$_1$-prelabeled for different periods of time (20, 60, and 180 min) after which they were subjected for 5 min at the indicated temperatures. Lipids were then extracted, separated by TLC, and visualized by autoradiography. (B) Quantitation by phoshoimaging of $^{32}$P-PtdOH formed at different temperatures in seedlings. (C) Formation of $^{32}$P-PtdOH in leaf disks at different temperatures. Values are means of triplicates ±SD. Asterisks indicate highest temperatures giving rise to a significant ($p < 0.05$) increase in $^{32}$P-PtdOH.

**FIGURE 2** | Temperature-dependent accumulation of $^{32}$P-PtdOH in *Arabidopsis* leaves and seedlings. (A) O/N $^{32}$P-prelabeled seedlings were incubated for 5 min at the indicated temperatures. Lipids were then extracted, separated by TLC, and visualized by autoradiography. (B) Quantitation by phoshoimaging of $^{32}$P-PtdOH formed at different temperatures in seedlings. (C) Formation of $^{32}$P-PtdOH in leaf disks at different temperatures. Values are means of triplicates ±SD. Asterisks indicate highest temperatures giving rise to a significant ($p < 0.05$) increase in $^{32}$P-PtdOH.

agreement with the results of the transphosphatidylidation assay (Figure 4A). Hence, the increase in $^{32}$P-PtdOH is unlikely to reflect a PLD activity, and is consistent with a DGK activity. At longer prelabeling time points, the relative increases in $^{32}$P-PtdOH gradually diminished (Figure 4C), due to the decrease in the specific radioactivity of the ATP pool and the general increase in structural phospholipid labeling.

Two of the most abundant PLD isoforms in Arabidopsis, PLDα1 and PLDδ8, have been implicated in cold stress tolerance (Ruelland et al., 2002; Li et al., 2004; Rajashekar et al., 2006). To test their contribution to the early PtdOH response to cold stress, both single and double KO-mutants were analyzed (Bargmann et al., 2009a,b). As shown in Figure 4D, all mutants exhibited a normal PtdOH response upon cold stress.

Together, these results argue against the involvement of PLD in the acute cold-shock-induced PtdOH response and strongly point to a role for DGK.

The implication of DGK in the early COR raised the question of DAG’s metabolic origin. One possible source of DAG is the induced PLC hydrolysis of the polyphosphoinositides, PtdInsP and/or PtdInsP$_2$, a well-defined plant stress response, which was supported by the observation that $^{32}$P-PtdInsP decreased in response to cold (Figure 2A). Moreover, this decrease correlated closely with an equivalent increase in $^{32}$P-PtdOH, in a temperature- and time-dependent fashion (Figure 5). These results strongly argue for the scenario that cold stress activates
PLC hydrolysis of PtdInsP to form DAG, which is subsequently phosphorylated to PtdOH by DGK.

PtdOH responses in Arabidopsis dgk- and cold stress mutants

Arabidopsis contains 7 DGK encoding genes. In an attempt to identify the isozyme involved in the cold-induced PtdOH response, a reversed genetic approach was used, screening a series of T-DNA insertion lines (Tables 1, 2). These lines carry insertions in or near the DGK encoding regions, although not all of the lines were established as knockout or knockdown mutants (Table 1, Figure A4). Seedlings of these lines were...
phosphoimaging. PtdOH levels are expressed as a percentage of the total each (32P-labeled lipids. Values are averages and quantified by phosphoimaging. PtdOH levels are expressed as percentage ∗ wild type (Tukey HSD test, 

increases were found in cold-stressed normal control PtdOH levels, neither showed significant defects sure their PtdOH response. Surprisingly, among the lines with 

dgk5-1

dgk4-1

dgk2-1

dgk2-2

dgk4-1

dgk4-2

dgk5-1

dgk6-1

dgk7-1

dgk7-2

dgk7-3

5-days-old Arabidopsis seedlings were labeled O/N with 32Pi and incubated at 0°C for 5 min. Lipids were then extracted separated by TLC and quantified by phosphoimaging. PtdOH levels are expressed as a percentage of the total 32P-labeled lipids and values represent averages of multiple samples containing 2 seedlings each (± SD).

* Transcript analysis by RTPCR confirmed knock-down (KD) or knock-out (KO) status. Expression of DGK6 was too low for detection hence the status of dgk6-1 could not be determined.

** Reproducible statistically significant difference of T-DNA line compared with wild type (Tukey HSD test, P < 0.05) within the wild type control homogeneous subset.

5-days-old Arabidopsis seedlings were labeled O/N with 32P, and incubated at 0°C for 5 min. Lipids were then extracted separated by TLC and quantified by phosphoimaging. PtdOH levels are expressed as a percentage of the total 32P-labeled lipids and values represent averages of multiple samples containing 2 seedlings each (± SD).

** Reproducibly statistically significant difference of T-DNA line compared with wild type (Tukey HSD test, P < 0.05) within the wild type control homogeneous subset.

| Gene   | AGI ID   | Line         | Name | Status* | Control | Cold | Fold increase | Replicates |
|-------|----------|--------------|------|---------|---------|------|---------------|------------|
| AtDGK1| At5g07920| SALK 053412  | dgk1-1 KO | 0.93 ± 0.07 | 3.11 ± 0.31 | 3.4 | 6 |
| AtDGK2| At5g63770| SAIL 718_G03 | dgk2-1 KO | 1.00 ± 0.06 | 2.70 ± 0.48 | 2.7 | 6 |
| AtDGK4| At2g20900| SAIL 339_C01 | dgk4-1 no KO | 1.22 ± 0.08 | 3.73 ± 0.26 | 3.1 | 3 |
| AtDGK5| At2g20900| SAIL 121_E10 | dgk5-1 KO | 0.85 ± 0.07 | 3.73 ± 0.15 | 4.4 | 3 |
| AtDGK6| At4g28130| SALK 016285  | dgk6-1 ND | 1.08 ± 0.03 | 4.48 ± 0.55 | 4.1 | 3 |
| AtDGK7| At4g30340| SAIL 51_E04  | dgk7-1 KO | 0.90 ± 0.17 | 2.92 ± 0.22 | 3.2 | 8 |
|        |          | SALK 059060  | dgk7-2 KO | 0.91 ± 0.14 | 3.24 ± 0.15** | 3.6 | 8 |
|        |          | SALK 007896  | dgk7-3 no KO | 0.87 ± 0.14 | 2.94 ± 0.19 | 3.4 | 8 |

In Arabidopsis, several COR mutants have been identified, including ice1, snow1, fry1, hos1, and los1 ([Ishitani et al., 1998; Xiong et al., 2001; Guo et al., 2002; Chinnusamy et al., 2003] mutation has been demonstrated to negatively affect cold-induced gene transcription by interfering with the function of AtICE1, a myc-type transcription factor which functions in CBF transcription in cold signaling. The myb-type transcription factor SNOW1/MYB15, also binds to the CBF promoter region, interacting with ICE1 (Agarwal et al., 2006).

To gain information on the position of the PtdOH response in the cold sensing pathway, each mutant was analyzed for its cold-induced PtdOH response. As shown in Figure 6A, fry1, hos1, and los1 all showed a normal response, but snow1 had a lower basal and cold shock-induced level of 32P-PtdOH (p = 0.006; Figure 6B); nevertheless, the relative stimulation levels were not significantly altered. Although the cold-induced PtdOH response in ice1 generally appeared to be lower than wild-type (Figure 7), it did not reach the significance level and was not cold-specific either, because the PtdOH response induced by salt stress (300 mM NaCl, 15 min) was also decreased (p = 0.008). Since these seedlings look stunted, pleiotropic effects are most likely to account for the observed differences. Similarly, PtdOH levels in the overexpressor of ICE1, Super-ICE1, tended to be suppressed, again indicating pleiotropic effects (Figure 7). Together these results indicate that the PtdOH response is upstream.

DISCUSSION

While, in Arabidopsis seedlings and leaves, the acclimation process in response to low temperatures is rapidly initiated, it takes 6–7 days to achieve maximal freezing tolerance (Uemura et al., 1995; Feng et al., 2007). The formation of PtdOH has been speculated to function in the regulation of this response (Ruelland et al., 2002; Xiong et al., 2002; Gomez-Merino et al., 2004; Li et al.,
Arisz et al. Cold-induced phosphatidic acid formation

FIGURE 6 | Cold-induced $^{32}$P-PtdOH induction in known Arabidopsis cold response mutants. Five-days-old seedlings were prelabeled O/N with $^{32}$Pi and subsequently incubated at 0°C or kept at room temperature for 15 min. Lipids were then extracted, separated by TLC, and quantified by phosphoimaging. $^{32}$P-PtdOH levels are expressed as percentage of the total $^{32}$P-lipid. Values are means from triplicate incubations from a typical experiment; error bars indicate SD. White bars, control; gray bars, 0°C.

(A) The mutants fry1, hos1, los1, and their wt background, C24 RD29A-LUC. (B) The snow1 mutant and the wt control, Col-0.

2004; Vergnolle et al., 2005; Rajashekar et al., 2006). While this has previously been studied in suspension-cultured cells, we have focused on the response in whole seedlings and mature leaves. The results showed that cold shock treatment triggered a rapid and sustained (during hours) accumulation of PtdOH, both in seedlings and in leaf discs of mature Arabidopsis plants (Figures 1–3). The leaf response was generally more pronounced, but in seedlings the PtdOH increase was faster and already visible upon minor temperature shifts which did not lead to a response in leaves. Since the accumulation of PtdOH is emerging as a common early element in environmental stress responses, and because it is suggested to be involved in the acclimation process, it is important to have knowledge of the underlying mechanisms.

DGK RATHER THAN PLD ACTIVITY GENERATES EARLY, COLD-INDUCED PtdOH

Two routes have been found to generate PtdOH under conditions of environmental stress in plants, i.e., PLD hydrolysis of structural phospholipids (i.e., PtdCho/PtdEtn/PtdGro) and phosphorylation of DAG by DGK (Arisz et al., 2009; Testerink and Munnik, 2011). Using transphosphatidylation assays, the absence of a $^{32}$P-PtdBut increase under chilling conditions that triggered massive $^{32}$P-PtdOH responses indicate that PLD is not involved (Figure 4A). Using a differential $^{32}$P-labeling assay, $^{32}$P-PtdOH demonstrated to be rapidly labeled, in agreement with a DGK involvement, and in contrast to the labeling of structural phospholipids PtdEtn, PtdCho, and PtdGro, which required long labeling times, again suggesting a PLD-independent pathway (Figure 4B).

Although this seemed at variance with studies of suspension-cultured cells, which suggested a cold-activated PLD activity (Ruelland et al., 2002; Cantrel et al., 2011), it is well-possible that PLD plays a role at a later phase of the COR. This is for example supported by (1) the induced membrane localization of PLDδ after 1 day at 2°C (Kawamura and Uemura, 2003), (2) its importance in freezing tolerance (Li et al., 2004; Chen et al., 2008; Du et al., 2010) and (3) the transcriptional regulation of Arabidopsis PLDδ and PLDα (Welti et al., 2002; Li et al., 2004) and two PLDα homologs from cotton (Kargiotidou et al., 2010) during cold acclimation. Nevertheless, consistent with the present data, we have found pldδ1/pldδ6 seedlings to display a normal $^{32}$P-PtdOH response after 5 min at 0°C (Figure 4D).

THE SUBSTRATE FOR DGK MAY BE GENERATED BY A PtdInsP-HYDROLYZING PLC

Since DGK was implicated, the question was raised how the substrate DAG was formed. Several pathways could account for this. A clue was provided by the concomitant decreases of $^{32}$P-PtdInsP, equivalent to the increase of $^{32}$P-PtdOH, suggesting the former to be precursor to DAG and PtdOH via PLC and DGK, respectively (Figures 5 and 8, reactions 1 and 3). Previously, cold stress in Arabidopsis cells has been shown to trigger decreases in both PtdInsP$_2$ and PtdInsP (Ruelland et al., 2002). While PtdInsP$_2$ is usually considered as the substrate for

FIGURE 7 | $^{32}$P-PtdOH responses in seedlings of the ice1 mutant and ICE1 overexpression transgenic line (Super-ICE1). Five-days-old seedlings were prelabeled O/N with $^{32}$Pi and incubated at 0°C with 300 mM NaCl for 15 min. $^{32}$P-PtdOH levels are expressed as percentage of the total $^{32}$P-lipid (average ±SD). $^{32}$P-PtdOH was enhanced due to cold and salt in all genotypes ($p < 0.025$), but salt-induced $^{32}$P-PtdOH was decreased in ice1 compared to wildtype (*$p = 0.008$).
In contrast, in both systems a knock-down allele of DGK7 mutations displayed an abrogation of the PtdOH response.DGK7 cold stress. Transgenically expressed but its transcript levels have not been found to increase upon seedlings (DGK2, Knockdown of DGK2, was associated with an increased accumulation of PtdOH. DGK1 kinase activity in cluster-I DGKs, (Brice et al., 2009). An the consumption of PtdIns4P to the generation of DAG through dephosphorylation of PtdIns4P by Sac1, coupling In yeast, the PtdIns substrate in this reaction is supplied in vitro shown to have OTHER POTENTIAL SOURCES OF DAG AND PtdOH

In young seedlings, but also in rosette leaves, DGK7 is more abundantly expressed than DGK1 and DGK2 (Arisz et al., 2009), but its transcript levels have not been found to increase upon cold stress. Transgenically expressed DGK7 protein has been shown to have in vitro DGK activity despite the lack of a C1 domain, which is thought to function in the regulation of kinase activity in cluster-I DGKs, DGK1 and DGK2 (Gomez-Merino et al., 2004, 2005; Arisz et al., 2009). DGK7 belongs to cluster II, together with DGK3 and DGK4 whose activity may be responsible for the enhanced PtdOH production in dgk7-2 seedlings. Recently, two interesting novel DAG sources have emerged in Arabidopsis stress responses: a PtdCho-hydrolyzing PLC, NPC4, that promotes tolerance to osmotic stresses (Peters et al., 2010), and SFR2, a galactolipid:galactolipid galactosyl transferase (GGGT), that produces DAG and oligogalactolipids to increase freezing tolerance (Thorlby et al., 2004; Moellering et al., 2010). For neither of the enzymes there is direct evidence that links their activity to stress-induced PtdOH accumulation. Rather, the enzymes could provide DAG as precursors for the synthesis of glycerolipids such as PtdCho and MGDG, or triacylglycerol, as for GGGT (Moellering et al., 2010; Moellinger and Benning, 2011). It will nevertheless be interesting to subject KO mutants and overexpression lines of the corresponding genes to a differential PtdOH screen as we have applied in this study.

PtdOH ACCUMULATION DUE TO COLD-INDUCED INHIBITION OF GLYCEROLIPID de novo SYNTHESIS?

Cold may have a direct impact on glycerolipid de novo synthesis as it lowers diffusion rates and decreases the number of substrate molecules that have sufficient energy to allow enzymatic reactions (Mahan et al., 2004). In fact, in our differential labeling experiments we noticed a cold-induced decrease in the rate of PtdEtn labeling, only visible after approximately 20–30 min of labeling when its de novo synthesis in seedlings can just be witnessed (Figure 4B). Also in leaf discs, 32P-PtdEtn labeling was decreased upon cold incubation, suggesting a cold-induced inhibition of PE's de novo biosynthesis (Figure A2). This effect occurred at temperatures ≤8°C, while the decrease remained constant at lower temperatures down to 0°C. In contrast, maximum 32P-PtdOH accumulation was achieved at 0°C (Figure A2).

Glycerolipid de novo synthesis starts with two acylations of Gro3P to generate PtdOH (Figure 8). For the synthesis of PtdIns (and PtdGro), PtdOH is converted to cytidine diphosphate-diacylglycerol (CDP-DAG), the substrate for phosphatidlyinositol synthase (PIS). Alternatively, PtdOH is dephosphorylated by phosphatidic acid phosphatase (PAP) to generate DAG as substrate in a reaction by which phosphoethanolamine (EtNP) is transferred from cytidine diphosphate-ethanolamine (CDP-Etn) to the lipid moiety, yielding PtdEtn. Cold does not seem to cause a general inhibition in the uptake of 32P, or its incorporation into the Kennedy pathway of glycerolipid de novo synthesis, since labeling of other structural phospholipids was not affected by cold stress (Figure A2). Therefore, the cause of decreased 32P-PtdEtn labeling is most likely in the synthesis or supply of its headgroup.

The precursor CDP-Etn is generated through the cytidylation of EtNP by phosphoethanolamine cytidylyl transferase (PECT), analogous to the PtdCho headgroup precursor CDP-Chol, being the product of phosphocholine cytidylyl transferase (CCT) using ChoP as substrate. The latter is produced by repeated methylations of EtNP, catalyzed by phosphoethanolaminemethyltransferase (PEAMT). This activity, which is considered rate-limiting for PtdCho synthesis, likely accounts for the different labeling kinetics of PtdCho and PtdEtn, only the latter being radioactively detected after 30 min of 32P1-labeling.
As previous studies have shown that low temperatures can inhibit the in vitro activity of recombinant CCT (Inatsugi et al., 2002), PECT activity is speculated to be similarly downregulated by cold (Figure 8, designated by “2”), resulting in a limited availability of CDP-Etn for PtdEtn synthesis. This would form a bottleneck leading to the accumulation of DAG as precursor for PtdEtn synthesis. Notably, this DAG could be an additional source for cold-induced PtdOH through DGK activity (Figure 8, reaction 3), which has been shown to be largely localized at the ER in Arabidopsis (Vaultier et al., 2008). Alternatively, accumulated DAG may block its own formation through feedback inhibition of PtdOH phosphatase (Figure 8, reaction 4), again promoting PtdOH accumulation. Similar regulation of PAP activity by product inhibition has been demonstrated in chloroplast envelope membranes from spinach (Malherbe et al., 1992).

In summary, we have shown a very fast (in minutes) accumulation of PtdOH in response to cold temperatures in Arabidopsis seedlings and leaf discs, which was not due to PLD activity. Instead, 32P-radiolabeling studies indicated a dominant role of DGK under these conditions. Using single T-DNA insertion lines, we were unable to pinpoint the DGK gene(s) involved but do propose that DGK acts in tandem with a PtdInsP-hydrolyzing PLC, based on the close correlation between the increase in 32P-PtdInsP and the decrease in 32P-PtdOH.

PtdOH accumulation was not affected by the fry1, hos1, and los1 mutations, consistent with an independent, upstream position in cold signaling. Although the snow1 and ice1 mutants displayed decreased PtdOH levels they likely reflected pleiotropic effects of the mutations.

Apart from the PLC/DGK route, additional, hypothetical sources of DAG and PtdOH were discussed, viz. via IPCS (Figure A1), NPC, GGGT and lipid de novo synthesis (Figure 8). Although for neither of these pathways there is sufficient evidence at present, they should not be ignored when studying PtdOH responses to cold or other environmental stresses.
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### Table A1 | Primers used for genotyping the DGK T-DNA insertion lines.

| Genotype  | LP          | RP          | Border primer | Sequence                  | Wild type     | Insertion   | LP/RP   | RP/LB   | Insertion |
|-----------|-------------|-------------|---------------|----------------------------|---------------|-------------|---------|---------|-----------|
| dgk1-1    | SALK_053412 | GGA TTC TCC TCC GTG AGA TTG | TCA TGC CGT ACT GGA AAA TTC | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 1205 bp | 588–888 bp | chr5 2527025 |
| dgk2-1    | SAIL_718_G03 | GCA AAG AAC AAA AAG GCA CAG | CAG ATG CA A GAC CGC TTT TAG | LB3              | TAGCATCTGAATTTCATAAC | LP/RP | RP/LB3 | 1106 bp | 439–739 bp | chr5 25520634 |
| dgk2-2    | SAIL_71_B03 | TGG TAC TGG ATC TGT TCG CC | CTA AAA GCG GTC TTG CAT CTG | LB3              | TAGCATCTGAATTTCATAAC | LP/RP | RP/LB3 | 957 bp | 432–732 bp | chr5 25520003 |
| dgk3-1    | SALK_082600 | TGC TCT CAG TGG GAA GAG ATC | CCG GAA AAC TAT CCG GTC AAC | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 1179 bp | 539–839 bp | chr2 8121795 |
| dgk4-1    | SAIL_339_C01 | AAA GCG AAG CCC ATA TAA AGC GAA GAG | GCC GAT GAT GGA TCG TGG TAG | LB3              | TAGCATCTGAATTTCATAAC | CAATCTGATACAC | LP/RP | RP/LB3 | 1011 bp | 447–747 bp | chr5 23371938 |
| dgk4-2    | SALK_069158 | TTT GAT CCC ATC GAA ACT CAC | GTC TTT GCG AAA TCG TGG TAG | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 1096 bp | 448–748 bp | chr5 23372207 |
| dgk4-3    | SALK_151239 | GGG AGC AGA ATC AGC AGG AAA | GAA TCA TCC TCG GAA TCA A ATG GAC | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 929 bp | ? | chr5 23374706 |
| dgk5-1    | SAIL_1212_E10 | TTC AGA GCA CAT GTG ACC AAC | TCC AAT TCG GAC ATT GTG TTC | LB3              | TAGCATCTGAATTTCATAAC | CAATCTGATACAC | LP/RP | RP/LB3 | 1163 bp | 505–805 bp | chr2 8990178 |
| dgk5-2    | SAIL_253_E12 | GAC TGG AGC TGT TGC TGA TCC | GGC CAA CAA TTT TGG TAG AAG | LB3              | TAGCATCTGAATTTCATAAC | CAATCTGATACAC | LP/RP | RP/LB3 | 1164 bp | 536–836 bp | chr2 8993229 |
| dgk6-1    | SALK_016285 | TGG GTA AAG TGA TCA ATG CAA AAG A | TGG CAA GCG AAA TGG GAA AGA | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 919 bp | ? | chr4 13973176 |
| dgk6-2    | SALK_054320 | GGG CCA TTA GTG GAA TAA AGC | CCA ATG CAA AAA CCT GAG | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 1265 bp | 606–906 bp | chr4 13971587 |
| dgk7-1    | SAIL_51_E04 | TTT GCA AGA ATG TTT CAA TTT TCC | TGC TGA TGG AGA TGG ACC TCC | LB3              | TAGCATCTGAATTTCATAAC | CAATCTGATACAC | LP/RP | RP/LB3 | 1118 bp | 434–734 bp | chr4 14839359 |
| dgk7-2    | SALK_059060 | TGA CAT ATA ACA CAC CAC ACC C | ACG ACC ACT TTT GCG GTC | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 904 bp | ? | chr4 14841224 |
| dgk7-3    | SALK_007896 | CTC CAG GAG TTT TAG TGT GGG | CCG AAC AGC TTT GGT TAA AGC | LBb1.3           | ATTTTGCCGATTTCGAAC | LP/RP | RP/LBb1.3 | 1056 bp | 457–757 bp | chr4 14839179 |
Table A2 | Primers used for RT-PCR expression analyses of DGK T-DNA insertion lines.

| Gene | Direction | Primename | Sequence                      |
|------|-----------|-----------|-------------------------------|
| DGK1 | sense     | SALK_053412_LP | GGATTCTCCTCCCGTAGATTG         |
|      | antisense | SALK_053412_RP | TCATGGCAATCTGGAAGACCTC        |
| DGK2 | sense     | dgk2 fw   | GACTGAGAGAGCTGAAACCC         |
|      | antisense | dgk2 rv   | GATGTTGATGGTTGAAAATTC        |
| DGK3 | sense     | SALK_082600_LP (dgk3-1) | TGCTTCAGTGGAAGAGATC          |
|      | antisense | dgk3 rv   | CAAAACTTTACCTCTACAAAC          |
| DGK4 | sense     | dgk4 fw   | GACATCTTCAGTTGAAAATTC        |
|      | antisense | dgk4 rv (A) | CCAAAGACTGTGGAAGGACTC        |
|      | antisense | dgk4 r3 (B) | CGATGTTGATGGTTGAAAATTC        |
| DGK5 | sense     | dgk5 fw2  | GACATCTTCAGTTGAAAATTC        |
|      | antisense | dgk5 rv   | GGAATCTTGAAGGTATCGGACAG      |
| DGK6 | sense     | dgk6 fw   | CTTGGAAACAGATAAGTCGG          |
|      | antisense | dgk6 rv   | CTTGGAAACAGATAAGTCGG          |
| DGK7 | sense     | SALK_007896_LP | CTCAAGGGAGTTTATGTTGGG     |
|      | antisense | dgk7 rv   | GTGTGTGTGTGTGTATCACCACAGCC  |
| SAND ref | sense   | At2g28390-Q-fw | CAGACAAAGCGCGATTTGGAATCA    |
|      | antisense | At2g28390-Q-rv | GCTTTCTCTCAAGGTGGTGGTGG      |

Sequences of the different primer pairs used to measure the expression of the various DGK genes used in this study. SAND (At2g28390) expression was used as the reference gene (Hong et al., 2010).

FIGURE A1 | Two pathways with the potential to generate DAG and PtdOH at the expense of PtdIns4P. PtdIns4P is suggested to be the substrate of cold-induced PLC activity which not only generates DAG, but at the same time releases InsP₂ that can be converted to InsP₆ and/or Ins. The latter products may have a functional relevance in the stress response because InsP₆ is a signaling compound in plants, and Ins is a precursor to compatible solutes (Vermeer and Munnik, 2010). Alternatively, inositolphosphorylceramide synthase (IPCS) generates DAG while transferring the InsP headgroup from PtdIns to ceramide (Cer), generating inositolphosphorylceramide (IPC). The PtdIns substrate in this conversion can be derived from PtdIns4P dephosphorylation, as, in yeast, through Sac1 activity. DAG generated via either of these pathways may subsequently be phosphorylated by DGK to generate PtdOH.

FIGURE A2 | Cold temperatures suppress ³²P-PtdEtn accumulation in Arabidopsis leaf disks. After 30 min ³²P-labeling and 5 min incubation at the indicated temperatures, phospholipids were quantitatively analyzed by phosphoimaging. Values are in arbitrary units (AU) representing means (±SD) of the radioactivity levels.
FIGURE A3 | Genotyping of the Arabidopsis dgk T-DNA insertion lines. DNA was isolated from leaf discs from 3 to 4 independent plants and zygosity determined by PCR using specific wt- and mutant-primer sets as listed in Table A1. Gel sample order (from left to right): Size marker; Col-0 (wt), with first primer set, LP/RP (= wt band) and second primer set, RP/LB (= insertion band); Three (or four) independent dgk plants, with first primer set, LP/RP (= wt band) and second primer set RP/LB (= insertion band); Last lane, primer control (−). Some gels end with the size marker. Results clearly show that all lines used are homozygous T-DNA insertion lines since Col-0 was the only line giving wt bands and all insertion lines gave only bands with the second primer set. For the SALK lines LBb1.3 was used as LB primer, for the SAIL lines LB3 was used as LB primer (see primer list, Table A1).
FIGURE A4 | Determination of DGK KO/KD-expression in Arabidopsis T-DNA insertion mutants by RT-PCR. RNA was isolated from 9-days old roots (DGK1,−3,−5,−7) or flowers (DGK2,−4,−6) since the latter genes did not reveal expression in the root (not shown). Predicted band sizes: DGK1, 702 bp; DGK3, 592 bp; DGK5, 534 bp; DGK8, 434 bp; DGK7, 791 bp; SAND (reference gene), 244 bp. Abbreviations: ND, not detectable; NAC, non-amplification control (test for genomic DNA contamination), i.e., RT reaction without RT enzyme on Col-0 RNA; NTC, no target control (test for contamination + primer dimers), i.e., RT reaction with water sample.

=> Summary:
dgk1-1 -> KD (exon)
dgk2-1 -> KO (exon)
dgk2-2 -> KO (exon)
dgk3-1 -> KO/KD (3’UTR)
dgk4-1 -> no KD (3’UTR)
dgk4-2 -> KO (exon)
dgk4-3 -> no KD (promotor)
dgk5-1 -> KO/KD intron)
dgk5-2 -> no KD (intron 5’UTR)
DGK6 -> ND
dgk6-1 -> possible KO/KD (exon)
dgk6-2 -> OE (exon, 40bp after ATG)
dgk7-1 -> strong KD (exon)
dgk7-2 -> weak KD (promotor)
dgk7-3 -> no KD (intron)