

# The Cold-Induced Early Activation of Phospholipase C and D Pathways Determines the Response of Two Distinct Clusters of Genes in Arabidopsis Cell Suspensions<sup>1[w]</sup>

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In plants, a temperature downshift represents a major stress that will lead to the induction or repression of many genes. Therefore, the cold signal has to be perceived and transmitted to the nucleus. In response to a cold exposure, we have shown that the phospholipase D (PLD) and the phospholipase C (PLC)/diacylglycerol kinase pathways are simultaneously activated. The role of these pathways in the cold response has been investigated by analyzing the transcriptome of cold-treated *Arabidopsis* (*Arabidopsis thaliana*) suspension cells in the presence of U73122 or ethanol, inhibitors of the PLC/diacylglycerol kinase pathway and of the phosphatidic acid produced by PLD, respectively. This approach showed that the expression of many genes was modified by the cold response in the presence of such agents. The cold responses of most of the genes were repressed, thus correlating with the inhibitory effect of U73122 or ethanol. We were thus able to identify 58 genes that were regulated by temperature downshift via PLC activity and 87 genes regulated by temperature downshift via PLD-produced phosphatidic acid. Interestingly, each inhibitor appeared to affect different cold response genes. These results support the idea that both the PLC and PLD pathways are upstream of two different signaling pathways that lead to the activation of the cold response. The connection of these pathways with the CBF pathway, currently the most understood genetic system playing a role in cold acclimation, is discussed.

During their development, plants are submitted to abiotic stresses, such as changes in light intensities, temperature conditions, and soil water potential, and to biotic stresses, such as interactions with microorganisms. Cold is one of the most widespread environmental stresses that has severe deleterious effects on plant function. Indeed, it lowers membrane fluidity and affects kinetic parameters and protein folding, thereby disturbing cytosolic and membrane processes. However, some plant species are cold tolerant, since they are able to respond and adapt to these changes.

Furthermore, the exposition to a moderate cold can trigger a physiological program named “cold hardening” that will allow some species to cope with freezing exposure. The development of cold tolerance and freezing tolerance correlates with changes in metabolite levels, such as the accumulation of Pro, sugars, and other cryoprotectants (Kaplan et al., 2004; Klotke et al., 2004). These cellular changes are in part the result of modifications in gene expression (Cook et al., 2004), with genes being up-regulated or down-regulated by cold temperatures. Recently, microarray technology has become a useful tool to analyze the changes in gene expression under cold stress (Seki et al., 2001, 2002; Chen et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Chinnusamy et al., 2003; Maruyama et al., 2004; Vogel et al., 2004). The cis-acting elements responsible for these changes in expression are being unraveled. An element, which has a 5-bp core sequence of CCGAC, designated the C-repeat (Baker et al., 1994) is often present in one to multiple copies in the promoters of many cold-regulated plant genes. This element, also identified as a dehydration-responsive element (Yamaguchi-Shinozaki and Shinozaki, 1994), is recognized by the CBF1, CBF2, and CBF3 proteins (Gilmour et al., 1998), also known as DREB1B, DREB1C,

<sup>1</sup> This work was supported by the Centre National de la Recherche Scientifique, the Université Pierre et Marie Curie, and the Ministère de l'Enseignement Supérieur et de la Recherche.

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<sup>[w]</sup> The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at [www.plantphysiol.org/cgi/doi/10.1104/pp.105.068171](http://www.plantphysiol.org/cgi/doi/10.1104/pp.105.068171).

and DREB1A, respectively (Liu et al., 1998). The transcript levels for all three *CBF* genes increase within 15 min of transferring plants to low temperature, after which the CBF proteins trigger the induction of target genes (Gilmour et al., 1998; Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2004). The cold induction of *CBF* depends on the action of factors, such as ICE1 (Chinnusamy et al., 2003), that bind to specific cis-elements present within CBF promoters (Zarka et al., 2003). However, not all cold-responsive genes belong to the CBF regulon, and Vogel et al. (2004) estimated that 70% of the cold-induced genes remained unassigned to any regulon.

In parallel to these studies of cold-induced transcriptome changes, much interest has been devoted to signaling pathways transducing the cold signal within the plant cell. We have shown that a cold treatment induces an increase of phosphatidic acid (PtdOH) within the first minutes of cold exposure to *Arabidopsis* (*Arabidopsis thaliana*) suspension cells (Ruelland et al., 2002). This was due to the activation of a phospholipase D (PLD) activity, as shown by transphosphatidylated, a reaction typical of PLD. But PtdOH also resulted from the action of phospholipase(s) C (PLC) coupled with a diacylglycerol kinase (DAGK) activity, as shown by the monitoring of inositol tris phosphate and by the effect of a DAGK inhibitor. The activation of PLC and PLD pathways were dependent on external calcium (Ruelland et al., 2002). These observations led us to ask the following questions with respect to the role of the activation of PLC and PLD in the cold response. Did PLC and PLD activation lead to a gene response, and if so, was it a positive or negative effect, activating or switching off gene expression? Which genes were cold responsive via the activation of PLC or PLD? In *Arabidopsis*, PLD, PLC, and DAGK are multi-gene families containing 12, 7, and 7 members, respectively (Elias et al., 2002; Mueller-Roeber and Pical, 2002; Qin and Wang, 2002; Gomez-Merino et al., 2004). The isoforms of PLD, PLC, or DAGK responsible for the activity detected early after a temperature downshift are unknown, although some of them have been shown to be up-regulated in response to a temperature downshift. For instance, *AtDAGK2*, *PLD $\alpha$* , and *PLD $\delta$*  are up-regulated by cold (Welti et al., 2002; Gomez-Merino et al., 2004; Li et al., 2004), and plants mutated in *PLD $\delta$*  are impaired in the development of freezing tolerance (Li et al., 2004). However, the fact that an isoform is up-regulated in response to a stress does not mean that it is the one responsible for the early transduction of this stress. To overcome this problem, we have applied a pharmacological approach using *Arabidopsis* suspension cells as a model. PLC activity was inhibited by U73122. As for PLD activity, adding ethanol to the cell medium, we were able to shift PtdOH production by PLD toward the production of phosphatidylethanol. However, in this case, it is important to note that ethanol does not inhibit the PLD-catalyzed hydrolysis of phospholipids but, being a substrate, reduces the production of the physiological signaling product, PtdOH, while

promoting the formation of a new phospholipid. We monitored transcriptome changes in response to a cold exposure in the presence of these agents modifying PLC and PLD pathway activities. In this way, we were able to identify gene clusters that could be considered as dependent either on PLC activity or on PLD-produced PtdOH for their cold response. These clusters were mainly characterized by a positive action of PLC or of PLD-produced PtdOH on cold response gene expression. Interestingly, it was found that pathways dependent on PLC activity or on PLD-produced PtdOH controlled the transcription of two different gene clusters. The role of the PLC and PLD pathways regarding the CBF regulon is discussed.

## RESULTS

### Time Course of Gene Induction in *Arabidopsis* Plantlets and Suspension Cells at 4°C

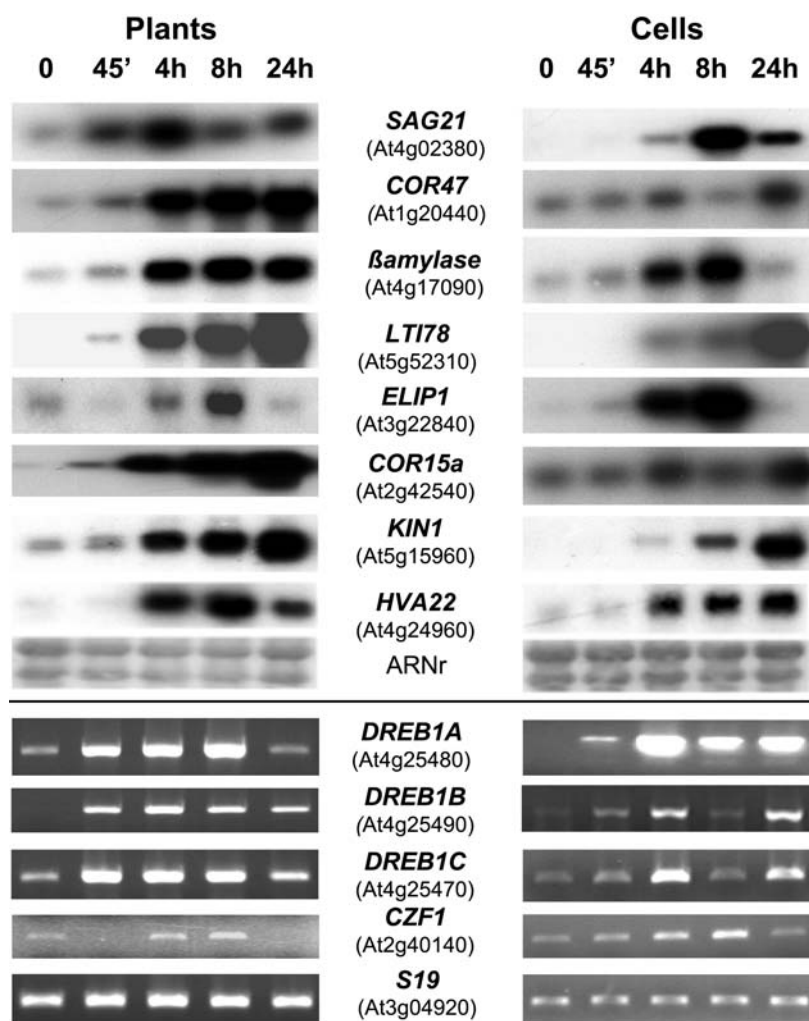
We first wanted to study the kinetics of gene induction by a cold shock in *Arabidopsis* cv Columbia suspension cells. We chose different genes that have been described as cold responsive in whole plants: *ELIP1*,  *$\beta$  amylase*, *SAG21*, *COR47*, *COR15A*, *LT178*, *KIN1*, *HVA22*, *DREB1A/CBF3*, *DREB1B/CBF1*, *DREB1C/CBF2*, and *CZF1* (Gilmour et al., 1998; Seki et al., 2001; Fowler and Thomashow, 2002). We followed their expression in plantlets and in suspension cells 45 min and 4, 8, and 24 h after transfer from 22°C to 4°C (Fig. 1).

The chosen genes were responsive to the cold treatment in plantlets and in suspension cells. However, the response kinetics were not always the same between the two models. The *DREB* factors appeared to display biphasic kinetics in cells, with a first peak at 4 h followed by a depression in RNA levels at 8 h and another peak at 24 h. Such kinetics were not detected in plantlets, where a peak was rapidly attained before slowly decreasing. Occasionally, the relative induction intensity was also different between plantlets and suspension cells, as illustrated by *COR15A*, for which the induction was more important in plantlets than in cells. On the other hand, *ELIP1* was more responsive in cells when compared to plantlets.

At this stage, our results indicated that *Arabidopsis* suspension cells responded to cold at the level of gene expression and that the CBF regulon, the currently most understood cold response regulon, was induced.

### Transcriptome Analysis after 4 h at 4°C

In order to get a broader view of gene induction/repression in response to a cold shock, we performed a microarray experiment with the complete *Arabidopsis* transcriptome microarray (CATMA) chip containing 24,715 probes, representing approximately 22,000 genes (Crowe et al., 2003; Hilson et al., 2004). RNAs were extracted from 6-d-old suspension cells either non-exposed to cold (22°C) or exposed to 4°C for 4 h. The RNAs from three independent biological repetitions



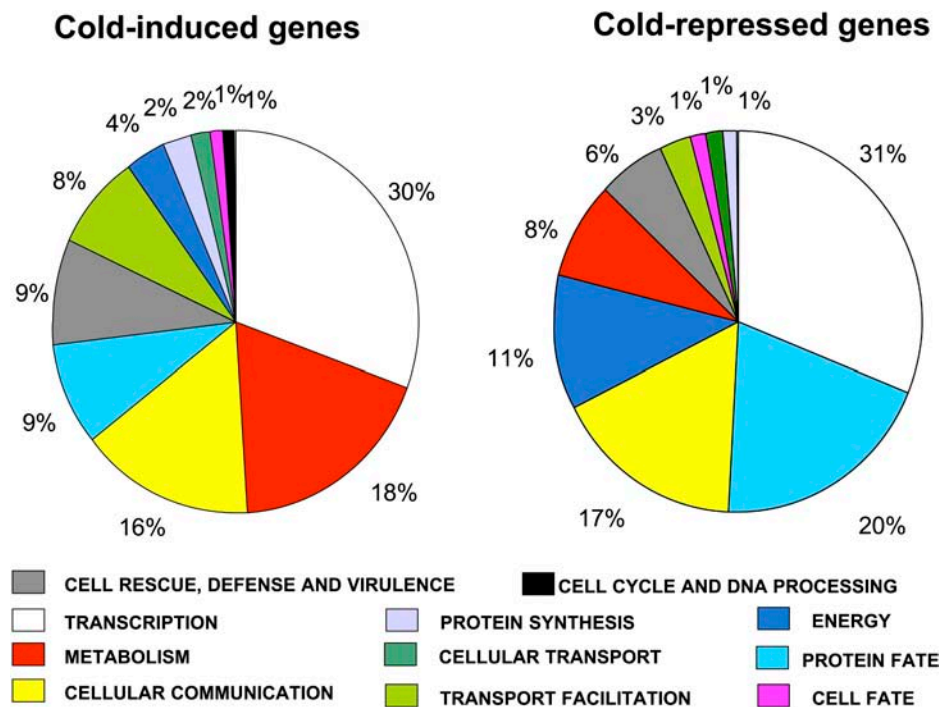
**Figure 1.** Gene expression in response to cold in Arabidopsis suspension cells and plantlets. Plants or cell suspensions cultivated at 22°C were exposed at 4°C for different time periods. RNA was isolated and submitted either to RNA-blot hybridization or to RT-PCR. For RNA-blot hybridization, gene-specific probes were used, and rRNA was used as a loading control. For RT-PCR, gene-specific primers were used, with the number of cycles optimized for each primer pair. S19 was used as a control.

were pooled and used as the matrix for the synthesis of fluorescent cDNA probes, permitting the comparison on the same slide with two independent two-color hybridizations (one dye-swap). The differentially expressed genes were identified by a paired *t* test on the log ratios. The raw *P* values were adjusted by the Bonferroni methods, which control the familywise error rate (FWER). With an FWER set at 5%, most of the probes, i.e. 24,059 probes (97.2% of total monitored probes), showed no changes in their transcript level. Nevertheless, 511 genes (2% of total monitored genes) could be considered as up-regulated, while 187 (0.8%) were down-regulated. When compared to other reports on gene expression in response to cold (Seki et al., 2001, 2002; Chen et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Chinnusamy et al., 2003; Maruyama et al., 2004; Vogel et al., 2004), our study describes 100 new genes that were cold repressed and 324 new genes that were cold induced. The list of the differentially regulated genes is available as Supplemental Table I.

Genes that were either up-regulated or down-regulated in response to cold were classified accord-

ing to the Munich Information Center for Protein Sequences (MIPS) functional catalog categories (Schoof et al., 2004), using the MIPS interface (<http://mips.gsf.de/proj/thal/db/index.html>; Fig. 2). Selected examples of cold-induced genes and cold-repressed genes are described in Tables I and II, respectively. In both cases, the most represented category was transcription (Fig. 2), indicating that after 4 h at 4°C, a major change in the transcriptome is still under way. The second most represented category differs between up-regulated and down-regulated genes, being metabolism and protein fate, respectively. The third most represented category was cellular communication in both cases, meaning that a readjustment of the cell signaling machinery is necessary for cold adaptation. The next gene categories that were up-regulated by cold exposure (in decreasing order) are protein fate, cell rescue, defense, and virulence, transport facilitation, energy, protein synthesis, cellular transport, cell fate, and cell cycle and DNA processing. For the genes that were down-regulated, the order is energy, metabolism, cell rescue, defense, and virulence, transport facilitation, cell fate, cellular transport, and protein synthesis.

**Figure 2.** Functional categories of the cold-induced genes or the cold-repressed genes. Genes that were up-regulated or down-regulated during a 4-h exposure at 4°C according to the microarray analysis were classified into functional categories using the MIPS interface (<http://mips.gsf.de/proj/thal/db/index.html>).



A cold exposure at 4°C for 4 h induced changes in gene expression that could be monitored by large-scale transcriptome analysis. We then wanted to take advantage of such a high-throughput analysis to determine which cold-responsive genes were dependent on activation of a PLC pathway, a PLD pathway, or both. To do so, we used various chemicals that interfere with these enzymes.

#### Effects of Phospholipase Inhibitors

Primary alcohols can be used as substrates by PLD, in the so-called transphosphatidylation reaction. In the presence of a primary alcohol, PLD will use it instead of water, leading to the formation of a phosphatidylalcohol instead of PtdOH. Secondary or tertiary alcohols are not substrates of this transphosphatidylation reaction (Munnik et al., 1995). We checked the effect of ethanol and tertiary butanol (tertButOH) on the production of PtdOH by PLD after a cold shock. Phospholipids were labeled by [<sup>33</sup>P]-orthophosphate, and radioactive ATP was quenched by an excess of non-radioactive phosphate added 15 min before the cold exposure. In such conditions, because ATP is no longer radioactive but the substrates of PLD are, radioactive PtdOH can only be produced by PLD (Ruelland et al., 2002). An exposure of 10 min at 0°C led to a 50% increase of the PtdOH level, reflecting PLD activity (Fig. 3A). When 0.9% (v/v) ethanol was added 15 min before the cold shock, the radioactive PtdOH level decreased to the level of nonstressed cells, while preincubation with 0.9% (v/v) tertButOH had no significant effect on PtdOH production (Fig. 3A).

Concomitantly, when ethanol was present, the appearance of phosphatidylethanol in cells stressed at 0°C confirmed that the PLD pathway was active. Besides, because primary and secondary alcohols have been shown to be able to activate PLD activity, maybe via G protein activation (Munnik et al., 1995), we checked whether the concentrations used did not lead to PLD activation. Cells were labeled for at least 12 h with <sup>33</sup>Pi, allowing a good labeling of putative PLD substrates. We then added to the cell medium 0.1% (v/v) primary butanol, together or not with 0.9% (v/v) ethanol or 0.9% (v/v) tertButOH. Lipids were extracted 10 min after the addition of the chemicals, and separated. As a control, cells treated with 0.1% (v/v) primary butanol were exposed for 10 min to 0°C or to 0.4% (v/v) 2-phenyl-2-propanol, an alcohol known to activate PLD. The production of phosphatidylbutanol was measured as a marker of PLD activity via its transphosphatidylation reaction. Our results (Supplemental Fig. 1) clearly showed that no increase in PLD activity could be seen when cells were treated with 0.9% (v/v) ethanol or 0.9% (v/v) tertButOH. In response to cold, an increase in PLD was detected, as it was in the presence of 2-phenyl-2-propanol. The fact that 0.9% (v/v) ethanol did not activate any PLD activity was confirmed when we monitored the level of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) in treated cells. Cells were labeled for at least 12 h with <sup>33</sup>Pi, and 0.9% (v/v) ethanol or 0.9% (v/v) tertButOH was added to the cell medium. Lipids were extracted at different times after alcohol treatment and separated by thin-layer chromatography. PtdCho and PtdEtn were quantified and their levels normalized to

**Table 1.** List of selected genes induced by a cold treatment

Genes have been classified by functional categories. Log I:  $\log_2$  average signal intensity. Log Rat:  $\log_2$  ratio, a negative ratio indicates that the gene is overexpressed in the conditions of cold stress. *P* values are Bonferroni corrected. Genes were considered as differentially regulated for a *P* value <5E-2. AGI, Arabidopsis Genome Initiative; ER, endoplasmic reticulum; ARF, ADP-ribosylation factor.

Processes	Examples	AGI	Log I	Log Rat	<i>P</i> Value
Transcription					
RNA transcription machinery	Transcription elongation factor-related	At5g11430	7.98	-1.11	3.15E-9
Transcription factors	AP2 domain containing factor	At1g68550	9.76	-1.83	0
Metabolism					
Polyamine biosyntheses	S-adenosylmethionine decarboxylase	At3g02470	9.44	-4.20	0
Other secondary metabolism	Phe ammonia-lyase	At3g10340	7.01	-1.19	7.13E-11
Lipid metabolism	Stearoyl-ACP desaturase	At2g43710	7.91	-2.24	0
Octadecanoid metabolism	Allene oxide synthase	At5g42650	7.94	-0.82	1.31E-3
Isoprenoid metabolism	Geranyl diphosphate synthase	At1g78510	7.52	-1.11	4.42E-9
Carbohydrate metabolism	Myo-inositol-1-P synthase	At4g39800	9.28	-1.66	0
Nucleotide metabolism	Amidophosphoribosyltransferase	At4g34740	8.97	-1.45	0
Amino acid metabolism	Cys synthase	At3g61440	7.92	-1.15	3.73E-10
Cellular communication					
Protein kinase	Ser/Thr protein kinase-like protein	At3g55450	8.62	-1.1	4.46E-9
Lipid signaling	PLD	At3g05630	7.07	-1.21	1.65E-11
Calcium signaling	Calcium-binding protein	At5g49480	7.23	-0.96	3.64E-6
Protein phosphatase	Protein phosphatase-2C	At4g03415	6.64	-0.74	2.17E-2
Protein fate					
Proteolytic degradation	Aminopeptidase	At4g36760	8.80	-2.24	0
Protein modification	Peptidylprolyl isomerase	At3g25230	7.12	-0.87	1.96E-4
Protein targeting	ER lumen protein retaining receptor	At2g21190	7.78	-0.73	2.48E-2
Protein folding	Chaperonin chain	At5g26360	7.70	-1.34	0
Cell rescue, defense, and virulence					
Detoxification	$\gamma$ -Glutamylcysteine synthetase	At4g23100	7.21	-0.75	1.52E-2
Defense	Class IV chitinase	At3g54420	7.60	-0.99	1.11E-6
Stress response	LTI30	At3g50970	8.40	-3.20	0
Transport facilitation					
Transport mechanism	ABC transporter family protein	At3g10670	7.48	-0.78	6.11E-3
Amino acid transporters	Amino acid permease	At2g38120	8.87	-0.74	1.81E-2
Carbohydrate transporters	Phosphate/triose-P translocator	At5g46110	7.34	-0.94	9.48E-6
Ion transporters	Mitochondrial phosphate translocator	At5g14040	7.21	-1.04	1.28E-7
Energy					
Respiration	Pyruvate decarboxylase	At4g33070	8.76	-1.34	0
Electron transport	NADH-ubiquinone oxidoreductase	At2g07717	9.66	-0.92	1.82E-5
Pentose-P pathway	6-Phosphogluconate dehydrogenase	At3g02360	7.70	-1.91	0
Glycolysis	Phosphoenolpyruvate carboxylase kinase	At1g08650	8.04	-1.10	5.88E-9
Protein synthesis					
Translation	Eukaryotic translation initiation factor	At3g26400	8.61	-0.83	9.88E-4
Ribosome biogenesis	Ribosomal protein L1 family protein	At2g42710	7.27	-0.96	3.42E-6
Cellular transport					
Extracellular transport	ARF protein	At3g53710	6.94	-0.93	1.59E-5
Vesicular transport	$\beta$ -Adaptin-like protein	At4g11380	7.91	-0.75	1.47E-2
Mitochondrial transport	Mitochondrial dicarboxylate carrier	At2g22500	9.64	-2.75	0
Cell fate					
Cell aging	Senescence-associated protein 5	At5g46700	8.36	-0.84	6.34E-4
Cell differentiation	Cell division protein FtsH-like protein	At3g02450	7.12	-0.95	7.67E-6
Cell cycle and DNA processing					
DNA processing	Histone deacetylase-like protein	At5g22650	8.34	-1.00	6.99E-7

that of phosphatidylinositol (Supplemental Fig. 2). For short times or longer times (up to 4h), no significant differences in PtdCho and PtdEtn levels could be detected, thus suggesting that no phospholipid hydrolysis was taking place.

U73122 is a commonly used PLC inhibitor, while U73343 is a less efficient analog used as a negative control. We labeled the cells for 2 h with  $^{33}\text{P}$ i. Under

these conditions, the radioactive PtdOH mainly comes from the PLC pathway (Ruelland et al., 2002). U73343 and U73122 were then added to the cells, and 15 min later, a 0°C shock was performed. The addition of 60  $\mu\text{M}$  U73122 led to a 50% decrease in the amount of PtdOH formed, indicating that PLC was inactivated. On the other hand, the inactive analog did not modify PLC activity (Fig. 3B).

**Table II.** List of selected genes repressed by a cold treatment

Genes have been classified by functional categories. Log I:  $\log_2$  average signal intensity. Log Rat:  $\log_2$  ratio, a positive ratio indicates that the gene is underexpressed in the conditions of cold stress. *P* values are Bonferroni corrected. Genes were considered as differentially regulated for a *P* value  $<5E-2$ . CBL, Calcineurin B-like protein; bZIP, basic-Leu zipper.

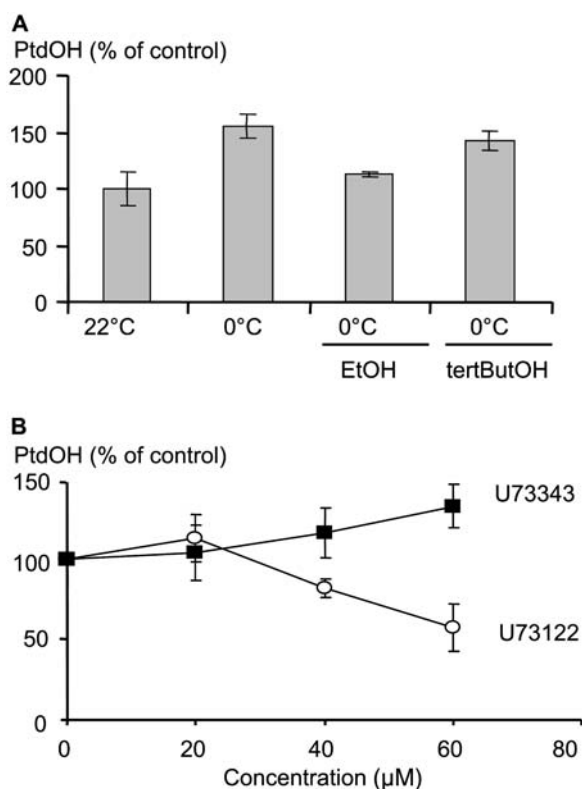
	Examples	AGI	Log I	Log Rat	<i>P</i> Value
Transcription					
Transcription factors	bZIP family protein	At5g49450	9.77	1.23	5.49E-12
	Myb family protein	At2g16140	9.04	1.01	3.73E-7
Chromatin modification	GCN5-related <i>N</i> -acetyltransferase	At5g16800	8.67	0.77	8.40E-3
Protein fate					
Proteolytic degradation	F-box family protein	At3g56470	7.30	1.01	3.76E-7
	SKP1 interacting partner	At3g61350	8.54	0.84	6.21E-4
Protein modification	Protein-L-isoaspartate <i>O</i> -methyltransferase	At3g48330	8.65	0.90	5.95E-5
Protein targeting	Peroxisomal targeting signal type 2 receptor	At1g29260	10.01	0.72	4.80E-2
Cellular communication					
Protein kinase	CBL-interacting protein kinase	At4g24400	8.00	0.72	4.10E-2
Lipid signaling	Inositol polyphosphate 5-phosphatase I	At1g34120	8.99	0.87	1.77E-4
Energy					
Photosynthesis	Photosystem II 5-kD protein	At1g51400	10.92	1.19	5.49E-11
	Photosystem II oxygen-evolving complex	At2g30790	8.09	1.01	4.55E-7
Electron transport	Chlorophyll <i>a/b</i> -binding protein	At3g08940	11.36	0.75	1.29E-2
	Thioredoxin	At1g76080	10.36	1.01	4.31E-7
	Glutaredoxin	At1g03850	8.03	1.09	9.16E-9
Metabolism					
Nucleotide metabolism	CTP synthase	At1g30820	8.99	0.80	2.64E-3
Amino acid metabolism	Lys decarboxylase family protein	At5g06300	9.03	0.81	2.02E-3
Cell rescue, defense, and virulence					
Detoxification	Peroxidase family protein	At4g32320	8.19	0.79	3.93E-3
Stress response	Wound-responsive protein	At4g28240	10.38	0.91	3.08E-5
	Drought-responsive family protein	At5g26990	7.21	0.77	6.65E-3
Transport facilitation					
Ionic homeostasis	Magnesium transporter	At5g09690	6.88	0.74	2.29E-2
Cell fate					
Cell aging	Senescence-associated protein	At4g35770	10.48	1.31	0
Cellular transport					
Vesicular transport	Golgi SNARE protein	At2g36900	7.97	0.93	1.50E-5
Protein synthesis					
Ribosome biogenesis	40S ribosomal protein S17	At3g10610	9.55	0.76	1.08E-2

### Microarray Analysis: Genes Depending on PLD-Produced PtdOH

RNAs were extracted from 6-d-old cells submitted to four different conditions: (1) no stress (22°C), (2) exposure at 4°C for 4 h, (3) exposure at 4°C for 4 h in the presence of 0.9% (v/v) tertButOH (hereafter, 4°C<sup>tertButOH</sup>), and (4) exposure at 4°C for 4 h in the presence of 0.9% (v/v) ethanol (hereafter, 4°C<sup>ethanol</sup>). The RNAs of three independent biological repetitions were pooled and reverse transcribed either with Cy3-dUTP or Cy5-dUTP to perform a two-color hybridization with the CATMA chip. One dye-swap (i.e. two hybridizations) was carried out for each of the two following combinations: 4°C versus 4°C<sup>tertButOH</sup> and 4°C<sup>ethanol</sup> versus 4°C<sup>tertButOH</sup>.

Among the 698 genes that were differentially regulated in response to a temperature downshift, 93 showed a transcript level difference between 4°C<sup>tertButOH</sup> and 4°C<sup>ethanol</sup> and may be considered as responding to cold via PLD-produced PtdOH (Fig. 4). These genes are listed in Supplemental Table II. Of these 93 genes,

73 were up-regulated in response to cold, while 20 were down-regulated. Of the 73 genes that were up-regulated at 4°C, 72 (i.e. 99%) had a lower amount of transcripts at 4°C in cells treated with ethanol when compared to tertButOH, thus showing an inhibitory effect specific of primary alcohols. A single gene had a higher transcript level at 4°C when comparing ethanol with tertButOH-treated cells, thus showing an inducing effect of ethanol versus tertButOH. Of the 20 genes that were down-regulated at 4°C, 15 (i.e. 75%) had higher amounts of transcripts at 4°C when cells had been preincubated with ethanol compared to a preincubation with tertButOH, thus showing an inhibitory effect of ethanol versus tertButOH on this repression. Only 5 of the 20 genes (i.e. 25%) had lower transcript amounts at 4°C when cells had been preincubated with ethanol compared to tertButOH, thus showing an inducing effect of primary alcohols on the repression. When taken together, it appears that 87 genes out of 93 genes (i.e. 94%) showed a repressing effect of ethanol versus tertButOH on their response to cold (Fig. 4).



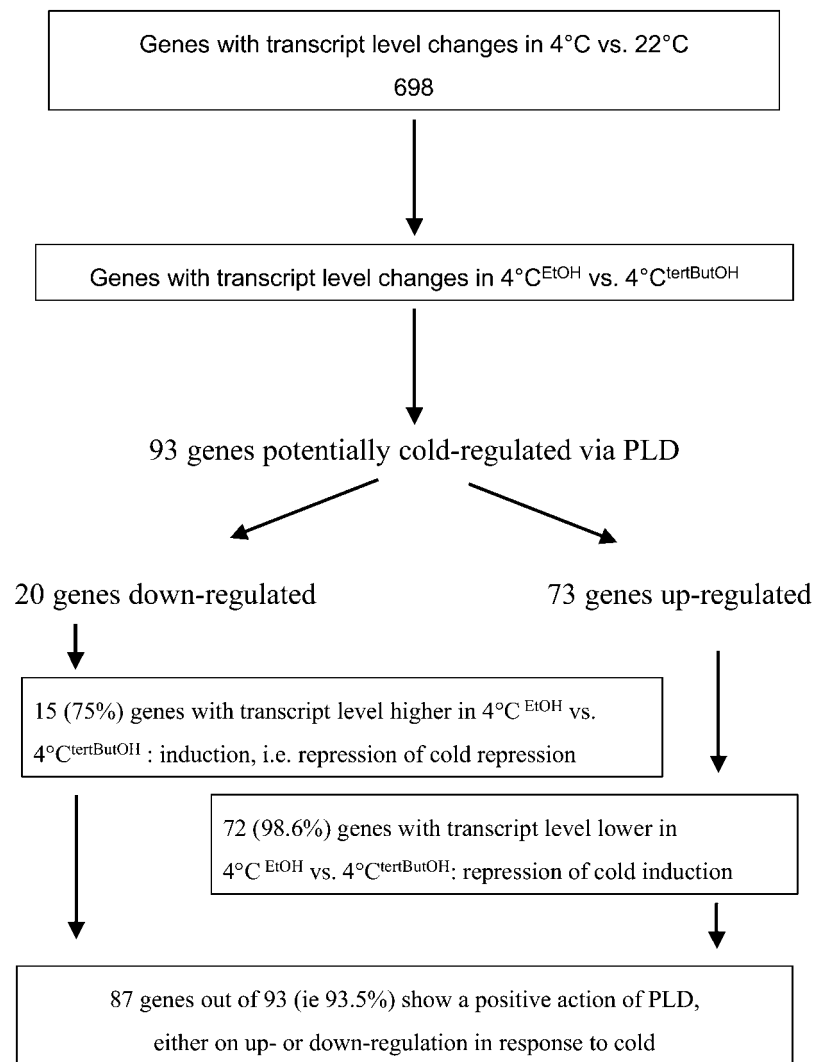
**Figure 3.** Inhibition of PLC/DAGK and PLD pathways. **A**, Cells were labeled in the presence of  $53 \text{ MBq L}^{-1} [^{33}\text{P}]\text{-PO}_4^{3-}$  for 2 h at  $22^\circ\text{C}$ . Different concentrations of U73122 or U73343 were added 15 min before cold shock. After a 10-min exposure at  $0^\circ\text{C}$ , lipids were extracted and separated by thin-layer chromatography. Radioactive PtdOH was first expressed as a percentage of total radioactive phospholipids and then normalized to the relative radioactive PtdOH content in the experiment with no chemicals. **B**, Cells were labeled in the presence of  $53 \text{ MBq L}^{-1} [^{33}\text{P}]\text{-PO}_4^{3-}$  for 24 h at  $22^\circ\text{C}$ , after which the cells were supplemented with 3 mM nonradioactive  $\text{PO}_4^{3-}$ . When necessary, 0.9% (v/v) ethanol or 0.9% (v/v) tertButOH was also added to the cell medium. Cells were submitted to a 10-min exposure at  $0^\circ\text{C}$  before lipids were extracted and separated using thin-layer chromatography. Radioactive PtdOH was expressed as a percentage of total radioactive phospholipids and then normalized to the relative radioactive PtdOH content in the experiment with no cold shock.

Is it valid to assign a gene to a pathway triggered by PLD-produced PtdOH based on both a cold responsiveness and an inhibition of ethanol versus tertButOH on that cold response? Statistical analyses support such an assignment. Genes could be classified into four clusters according to whether their expression was induced or repressed by cold and whether the response was inhibited or not by ethanol versus tertButOH (Table III). In our microarray analysis, 698 genes were differentially cold regulated. These genes were distributed as follows: 73% (i.e. 511 out of 698) were up-regulated by cold, while 27% (i.e. 187 out of 698) were down-regulated by cold. In the microarray analysis, 259 genes showed a difference in transcript level between  $4^\circ\text{C}^{\text{ethanol}}$  versus  $4^\circ\text{C}^{\text{tertButOH}}$ . These genes were distributed in the following way: 25% (i.e. 65 out of 259) had a transcript level higher in  $4^\circ\text{C}^{\text{ethanol}}$  versus

$4^\circ\text{C}^{\text{tertButOH}}$ , while 75% (i.e. 194 out of 259) had a transcript level lower in  $4^\circ\text{C}^{\text{ethanol}}$  versus  $4^\circ\text{C}^{\text{tertButOH}}$ . Therefore, for each of the four clusters, it was possible to calculate a theoretical number of genes based on the hypothesis that the difference in transcript level between  $4^\circ\text{C}^{\text{ethanol}}$  versus  $4^\circ\text{C}^{\text{tertButOH}}$  was independent of the transcript level difference between  $4^\circ\text{C}$  and  $22^\circ\text{C}$ . For instance, the cluster ( $4^\circ\text{C}^{\text{ethanol}} < 4^\circ\text{C}^{\text{tertButOH}}$ ) and ( $4^\circ\text{C} < 22^\circ\text{C}$ ) had a theoretical number of  $93 \times 0.75 \times 0.27$ , i.e. 18.83. The observed number of genes in each cluster was compared to this theoretical number (Table III). It is clear that there was an overrepresentation of genes showing a positive cold response action with respect to PLD-produced PtdOH (i.e. an inhibiting effect on the cold response). A  $\chi^2$  analysis indicated that these differences in distribution cannot be an effect of random events ( $P < 0.001$ ). Therefore, for the following analysis, the 87 genes that showed a repressing effect of ethanol versus tertButOH on their cold response were considered as driven by a pathway triggered by PLD-produced PtdOH (Supplemental Table II).

We wanted to know whether this regulation at  $4^\circ\text{C}$  by PLD-produced PtdOH could be correlated with a regulation already present at ambient temperature. To answer this question, RNA was extracted from 6-d-old cells submitted to three different conditions: (1) no stress ( $22^\circ\text{C}$ ), (2) exposure at  $22^\circ\text{C}$  for 1 h and 30 min in the presence of 0.9% (v/v) tertButOH (hereafter,  $22^\circ\text{C}^{\text{tertButOH}}$ ), and (3) exposure at  $22^\circ\text{C}$  for 1 h and 30 min in the presence of 0.9% (v/v) ethanol (hereafter,  $22^\circ\text{C}^{\text{ethanol}}$ ). The RNA of three independent biological repetitions was pooled and reverse transcribed either with Cy3-dUTP or Cy5-dUTP to perform a two-color hybridization with the CATMA chip. One dye-swap (i.e. two hybridizations) was made for each of the two following combinations:  $22^\circ\text{C}$  versus  $22^\circ\text{C}^{\text{tertButOH}}$  and  $22^\circ\text{C}^{\text{ethanol}}$  versus  $22^\circ\text{C}^{\text{tertButOH}}$ . Of the approximately 21,000 genes assayed, only 124 genes had a transcript level difference between  $22^\circ\text{C}^{\text{ethanol}}$  versus  $22^\circ\text{C}^{\text{tertButOH}}$  (Supplemental Table III). Among these 124 genes, 54 genes showed higher transcript levels in  $22^\circ\text{C}^{\text{tertButOH}}$  than in  $22^\circ\text{C}^{\text{ethanol}}$ , while 70 genes had higher transcript levels in  $22^\circ\text{C}^{\text{ethanol}}$  than in  $22^\circ\text{C}^{\text{tertButOH}}$ . These 124 genes represented genes that might have a regulation (either positively or negatively) for their basal expression by a basal PLD activity. Are the genes we assigned as being cold regulated via PLD-produced PtdOH also regulated by PLD-produced PtdOH for their basal expression? Within the 87 genes driven by PLD-produced PtdOH, only seven genes showed a transcript level difference between  $22^\circ\text{C}^{\text{tertButOH}}$  and  $22^\circ\text{C}^{\text{ethanol}}$ . For the seven genes that showed a difference in transcript level between  $4^\circ\text{C}^{\text{tertButOH}}$  versus  $4^\circ\text{C}^{\text{ethanol}}$  and between  $22^\circ\text{C}^{\text{tertButOH}}$  versus  $22^\circ\text{C}^{\text{ethanol}}$ , it was remarkable to observe that when a gene had a higher transcript level in  $4^\circ\text{C}^{\text{tertButOH}}$  than in  $4^\circ\text{C}^{\text{ethanol}}$ , the same gene had a lower transcript level in  $22^\circ\text{C}^{\text{tertButOH}}$  than in  $22^\circ\text{C}^{\text{ethanol}}$  (Supplemental Table II). Conversely, when a gene had a lower transcript

**Figure 4.** Determination of the genes downstream of the PLD pathway. Among the 861 genes that showed transcript level changes in response to 4°C versus 22°C or in response to 4°C<sup>ethanol</sup> versus 4°C<sup>tertButOH</sup>, 93 showed transcript level changes in response to both 4°C versus 22°C and to 4°C<sup>ethanol</sup> versus 4°C<sup>tertButOH</sup>. These genes are potentially cold regulated via the PLD pathway. Of these 93 genes, 20 were down-regulated by the cold treatment, while 73 were up-regulated.



level in 4°C<sup>tertButOH</sup> than in 4°C<sup>ethanol</sup>, it had a higher transcript level in 22°C<sup>tertButOH</sup> than in 22°C<sup>ethanol</sup>. Therefore, the genes that are up-regulated by cold via PLD-produced PtdOH are down-regulated at 22°C by PLD-produced PtdOH (via a basal PLD activity), and the genes that are down-regulated by cold via PLD-produced PtdOH are up-regulated at 22°C by PLD-produced PtdOH. However, the broad majority of the 87 genes driven by PLD-produced PtdOH (80 of 87) were not associated with a putative PLD regulation of their basal level. And for the seven genes that are, the effects of ethanol on gene expression at 4°C could not be attributed to a regulation already present at ambient temperature. Therefore, we consider that we have identified 87 genes that are cold regulated via PLD-produced PtdOH.

#### Microarray Analysis: Genes of the PLC Pathway

RNA was extracted from 6-d-old cells that had been submitted to four different conditions: (1) no stress

(22°C), (2) exposure at 4°C for 4 h, (3) exposure at 4°C for 4 h in the presence of U73122 (hereafter, 4°C<sup>U73122</sup>), and (4) cells exposed to 4°C for 4 h in the presence of U73343 (hereafter, 4°C<sup>U73343</sup>). RNA extracted from three independent biological repetitions was pooled and reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP to perform a two-color hybridization with the CATMA chip. One dye-swap (i.e. two hybridizations) was made for each of the two following combinations: 4°C versus 4°C<sup>U73343</sup> and 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup>. Among the 698 genes that were differentially regulated in response to a temperature drop, we considered the genes showing a transcript level difference between 4°C<sup>U73122</sup> and 4°C<sup>U73343</sup> (Fig. 5). However, some of these genes also showed a difference in transcript levels between 4°C<sup>U73343</sup> and 4°C. Since U73343 was dissolved in a dimethyl sulfoxide (DMSO)/tertButOH mix, the difference in transcript levels between 4°C<sup>U73343</sup> and 4°C could indicate a solvent effect on gene expression. Therefore, the effect of U73122 versus U73343 could be attributed to an effect of the solvents and not to a



**Table III.** Analysis of expected and observed clusters of genes classified by their cold response and the effect of ethanol versus tertButOH on this cold response

The expected number of genes for each cluster were calculated and compared to the actual number observed in our analysis. With respect to the expected numbers, the categories written in bold are over-represented in the experimental data, while the categories written in italics are underrepresented. For each category, these differences in distribution correlate to an inhibitory effect of ethanol versus tertButOH on the cold response.

PLD	4°C > 22°C		4°C < 22°C	
	<i>4°C<sup>ethanol</sup> &gt;</i>	<i>4°C<sup>ethanol</sup> &lt;</i>	<i>4°C<sup>ethanol</sup> &gt;</i>	<i>4°C<sup>ethanol</sup> &lt;</i>
	<i>4°C<sup>tertButOH</sup></i>	<i>4°C<sup>tertButOH</sup></i>	<i>4°C<sup>tertButOH</sup></i>	<i>4°C<sup>tertButOH</sup></i>
Theoretical	16.8	51.3	6.1	18.8
Observed	1	<b>72</b>	<b>15</b>	5

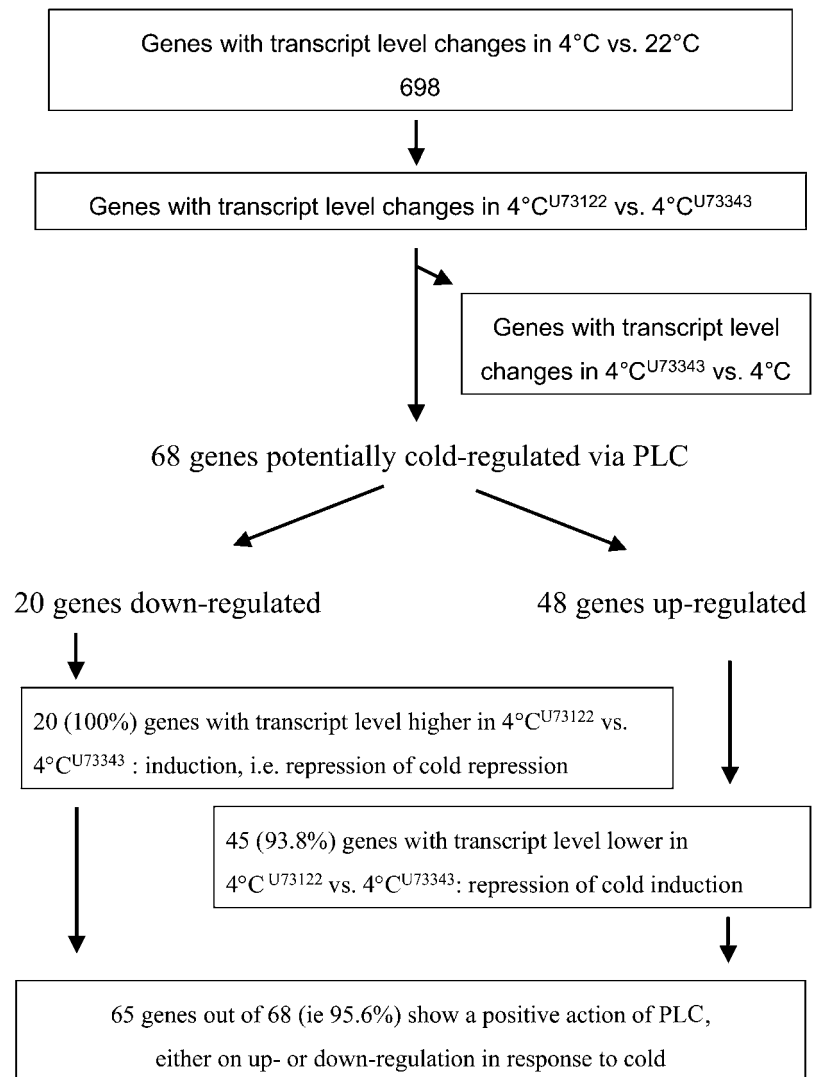
PLC-dependent cold activation. These genes were not considered in our analysis. The 68 remaining genes showed a transcript level difference between 4°C<sup>U73122</sup> and 4°C<sup>U73343</sup> and between 4°C and 22°C. These genes may be cold-regulated via a PLC pathway. They are listed in Supplemental Table IV. Of these 68 genes, 48 were up-regulated in response to cold, while 20 were down-regulated. Of the 48 up-regulated genes, 45 (i.e. 94%) showed lower transcripts levels at 4°C when cells had been preincubated with U73122 compared to U73343, thus showing an inhibitory effect of U73122 on the induction. All of the 20 genes that were down-regulated at 4°C had higher transcript amounts at 4°C when the cells had been preincubated with U73122 compared to U73343, thus showing an inhibitory effect of U73122 versus U73343 on the repression. When considered together, it appeared that 65 out of the 68 genes (i.e. 96%) showed a repressing effect of U73122 versus U73343 on their response to the cold treatment.

Again, we wanted to know whether it was valid to assign a gene to a PLC-triggered pathway based on both a cold responsiveness and an inhibition by U73122 versus U73343 on the cold response. Statistical analyses supported such assignments. We classified genes into four clusters according to whether the expression was induced or repressed by the cold and whether the cold response was inhibited or not by U73122 versus U73343 (Table IV). After removing from the analysis the genes that showed a difference between 4°C versus 4°C<sup>U73343</sup>, 433 genes appeared to be differentially cold regulated. These genes were distributed in the following manner: 70.4% (i.e. 305 out of 433) were up-regulated by cold, while 29.6% (i.e. 128 out of 433) were down-regulated. The 211 genes showing a differential transcript level between 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup> were distributed as follows: 65.9% (i.e. 139 out of 211) had a transcript level higher between 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup>, while 34.1% (i.e. 72 out of 211) had a lower transcript level between 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup>. Therefore, for each of the four clusters, it was possible to calculate a theoretical number of genes based on the hypothesis that the transcript level difference between 4°C<sup>U73122</sup> versus

4°C<sup>U73343</sup> was independent of the transcript level difference between 4°C versus 22°C. The observed number of genes in each cluster was then compared to this expected number (Table IV). It is clear that there was an overrepresentation of genes showing a positive action of PLC on the cold response (i.e. an inhibiting effect on the cold response) combined with an underrepresentation of genes not showing such a positive action. A  $\chi^2$  analysis indicated that these differences in distribution were not an effect of random events ( $P < 0.001$ ). Therefore, the 65 genes having the characteristics of genes that respond to cold via PLC were considered as PLC driven (Supplemental Table IV).

We wanted to know whether this regulation at 4°C by PLC activity was in a way correlated with a regulation already present at ambient temperature. To answer this question, RNA was extracted from 6-d-old cells submitted to three different conditions: (1) no stress (22°C), (2) exposure at 22°C for 1 h and 30 min in the presence of U73122 (hereafter, 22°C<sup>U73122</sup>), and (3) exposure at 22°C for 1 h and 30 min in the presence of U73343 (hereafter, 22°C<sup>U73343</sup>). The RNA of three independent biological repetitions was pooled and reverse transcribed either with Cy3-dUTP or Cy5-dUTP to perform a two-color hybridization with the CATMA chip. One dye-swap (i.e. two hybridizations) was made for each of the two following combinations: 22°C versus 22°C<sup>U73343</sup> and 22°C<sup>U73122</sup> versus 22°C<sup>U73343</sup>. Of the approximately 21,000 genes assayed, 1,506 genes had a transcript level difference between 22°C<sup>U73122</sup> versus 22°C<sup>U73343</sup> (listed in Supplemental Table V). Among these 1,506 genes, 734 genes showed higher transcript levels, while 772 genes had lower transcript levels at 22°C<sup>U73343</sup> when compared to 22°C<sup>U73122</sup>. These 1,506 genes represented genes that might have their basal expression regulated (either positively or negatively) by a basal PLC activity. Are the genes we assigned as being cold regulated via PLC also regulated by PLC for their basal expression? Among the 65 genes dependent on PLC activity, only seven genes showed a transcript level difference between 22°C<sup>U73122</sup> and 22°C<sup>U73343</sup> (Supplemental Table IV). Therefore, for the majority of the 65 identified genes, there is no ambiguity and the effect of U73122 versus U73343 can only be attributed to a cold-induced PLC activity. The genes that were up-regulated by cold and that were dependent for that regulation on PLC activity had a lower transcript level at 4°C<sup>U73122</sup> than at 4°C<sup>U73343</sup>. Most of these genes did not show any difference in transcript levels between 22°C<sup>U73122</sup> and 22°C<sup>U73343</sup>, while three genes had lower transcript levels at 22°C<sup>U73122</sup> versus 22°C<sup>U73343</sup>. Conversely, the genes that were down-regulated by cold and that were dependent for this regulation on PLC activity had higher transcript levels at 4°C<sup>U73122</sup> than at 4°C<sup>U73343</sup>. Most of the genes did not show any difference in transcript levels between 22°C<sup>U73122</sup> and 22°C<sup>U73343</sup>, but four genes had higher transcript levels at 22°C<sup>U73122</sup> versus 22°C<sup>U73343</sup>. Therefore, the vast majority of the 65 genes we assigned as PLC driven for their cold

**Figure 5.** Determination of the genes downstream of the PLC/DAGK pathway. Among the 1,172 genes that showed transcript level changes in response to 4°C versus 22°C or in response to 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup>, 591 genes showing a transcript level change in response to 4°C<sup>U73343</sup> versus 4°C were removed from the analysis. Of the remaining genes, 68 showed transcript level changes in response to 4°C versus 22°C and in response to 4°C<sup>U73122</sup> versus 4°C<sup>U73343</sup>. These genes are potentially cold-regulated via the PLC pathway. Of these 68 genes, 20 were down-regulated by the cold treatment, while 48 were up-regulated.



response did not show any sensibility to PLC inhibitors for their ambient expression. Only seven genes showed such a sensibility, since the effect of U73122 versus U73342 was the same at 4°C and at 22°C. Therefore, for these genes, we cannot be sure that the observed low temperature effect was not due to a regulation already existing at ambient temperature. For this reason, these seven genes are not included in the list of cold-responsive, PLC-driven genes. Nevertheless, we have identified 58 genes that are cold regulated via PLC activity: 42 being up-regulated and 16 showing down-regulation via PLC activity.

#### Confirmation by RT-PCR and Northern-Blot Analyses

The results obtained by microarray analyses were confirmed on a selection of genes by using various agents acting on phospholipase pathways. For genes driven by PLD-produced PtdOH, we tested different concentrations of ethanol (0%, 0.3%, 0.9%, and 1.8% [v/v]) and 0.7% (v/v) tertButOH as a control. North-

ern blots or RT-PCR were carried out with *SAG21*, *LTI78*, *HVA22*, *LTI30* (a Glu dehydrogenase encoding gene), *CZF1*, a gene encoding an AP2 domain containing protein, and *WRKY33*. A gene encoding a Myb factor, *MYB73*, which was not classified in the genes driven by PLD-produced PtdOH, was tested as a control; the gene (*At3g04920*) encoding the ribosomal protein S19 was used as a constitutive probe (Fig. 6). As expected, ethanol showed an inhibiting effect at 0.3% and at 0.9% (v/v). But for some genes, less inhibition was detected in the presence of 1.8% (v/v) ethanol (Fig. 6). This can be explained if the genes contain a cis-element in their promoter that is sensitive to G proteins, since they can be activated by this higher concentration of ethanol. The G protein activation would counterbalance the inhibiting effect of PLD by transphosphatidylation. The presence of ethanol had no effect on the expression of *MYB73* and *S19*, as expected.

For PLC-driven genes, we tested the cold response of *MYB73* and a gene encoding an AP2 domain containing protein. *LTI30* was used as a control, and

**Table IV.** Analysis of expected and observed clusters of genes classified by their cold response and the effect of U73122 versus U73343 on this cold response

The expected number of genes for each cluster were calculated and compared to the actual number observed in our analysis. With respect to the expected numbers, the categories written in bold are over-represented in the experimental data, while the categories written in italics are underrepresented. For each category, these differences in distribution correlate to an inhibitory effect of U73122 versus U73343 on the cold response.

PLC	4°C > 22°C		4°C < 22°C	
	4°C <sup>U73122</sup> > 4°C <sup>U73343</sup>	4°C <sup>U73122</sup> < 4°C <sup>U73343</sup>	4°C <sup>U73122</sup> > 4°C <sup>U73343</sup>	4°C <sup>U73122</sup> < 4°C <sup>U73343</sup>
	Theoretical	31.5	16.3	13.2
Observed	3	<b>45</b>	<b>20</b>	0

S19 was used as a constitutive probe. We tested the influence of U73122 and edelfosine, an inhibitor of PLC that inhibits the cold-induced production of InsP<sub>3</sub> (Ruelland et al., 2002). The effect of U73122 must be compared to the effect of U73343, while edelfosine treatment can be compared to the control, as this drug is dissolved in water. MYB73 and the gene encoding the AP2 domain protein showed an inhibiting effect of U73122 versus U73343. The inhibition of the cold induction of these genes by inhibiting PLC activity was confirmed by 150 μM edelfosine. LTI30 expression was not sensitive to the presence of inhibitors (Fig. 7).

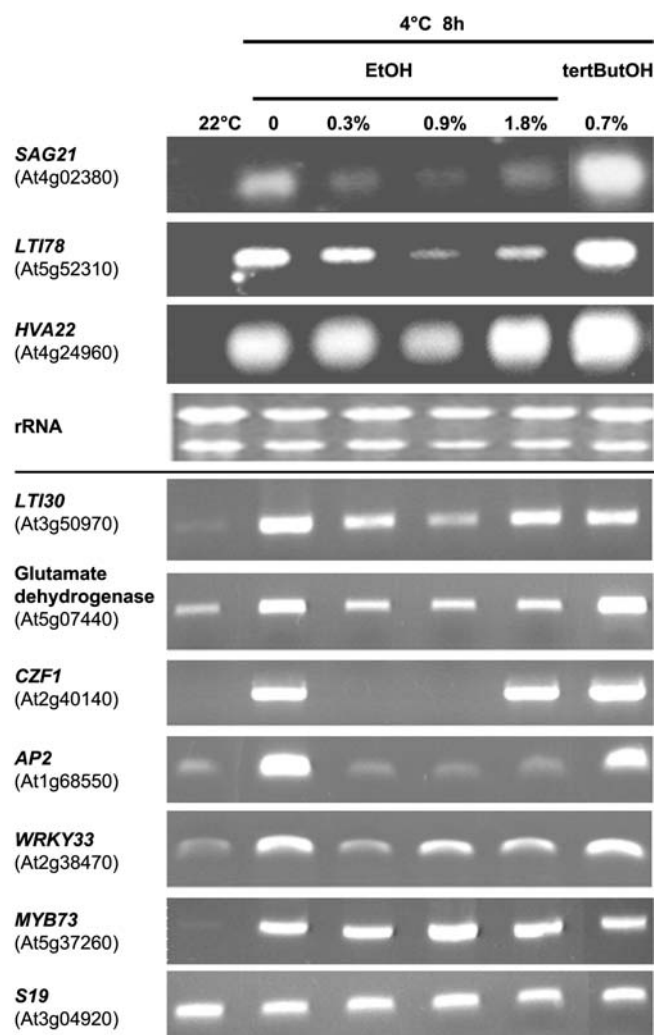
#### PLC and PLD Are Upstream of Two Distinct Gene Clusters

In order to determine whether some genes were dependent on both pathways, we compared the list of the cold response genes that depended either on PLC activity or on the production of PtdOH by PLD. Among the cold-induced genes, only seven were activated via both PLC and PLD (Table V). This means that among the genes that were cold induced via PLC, 83% were not cold activated via PLD-produced PtdOH and that 89% of the genes that were cold-induced by PLD-produced PtdOH were not cold activated via PLC. For the genes that were cold repressed, only one gene was repressed via both PLC activity and PLD-produced PtdOH. This strongly suggests that PLC activity and PLD-produced PtdOH are upstream pathways that regulate two different clusters of genes and that the signaling pathways triggered by the activation of PLC in response to cold and by PtdOH production by PLD in response to cold are distinct.

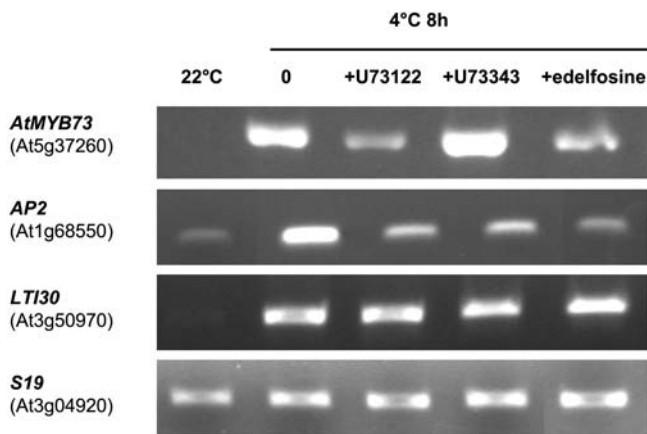
#### PtdOH and the CBF Regulon

The CBF/DREB1 pathway is currently the best understood genetic system playing a role in cold acclimation. A number of studies have looked for CBF/DREB1 target genes by over-expressing these proteins (Kasuga et al., 1999; Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2004). We searched whether our gene candidates being driven by

PLD-produced PtdOH or by PLC activity have been described as CBF targets. For genes up-regulated by cold, we found that 17 out of 511 (3.3%) are mentioned as CBF target genes in at least one of the reference studies (Supplemental Table I). When the 72 genes dependent on PLD-produced PtdOH for their cold activation are considered, the proportion of CBF targets rises to 12.5% (9 out of 72 genes). On the other hand, for PLC-dependent genes, only 3 out of 42 have been described as CBF target genes, i.e. 7.1%. No obvious overrepresentation can be concluded from these results on so few genes. Therefore, we checked the cold expression of *DREB1A* in the presence or absence of agents



**Figure 6.** Effect of inhibitors of the PLD pathway on gene induction in response to cold. RNA was isolated from 5-d-old cell suspensions cultivated at 22°C with or without exposure for 8 h at 4°C. In the case of a cold exposure, when indicated, the cell medium was supplemented with 0.3% (v/v), 0.9% (v/v), or 1.8% (v/v) of ethanol or with 0.7% (v/v) of primary butanol or 0.7% (v/v) of tertButOH. RNA was submitted either to RNA-blot hybridization or to RT-PCR. For RNA-blot hybridization, gene-specific probes were used, and rRNA was used as a loading control. For RT-PCR, gene-specific primers were used, with the number of cycles optimized for each primer pair. S19 was used as a control.



**Figure 7.** Effect of inhibitors of the PLC pathway on gene induction in response to cold. RNA was isolated from 5-d-old cell suspensions cultivated at 22°C with or without exposure for 8 h at 4°C. In the case of a cold exposure, when indicated, the cell medium was supplemented with U73122 (60  $\mu$ M), U73343 (60  $\mu$ M), or edelfosine (150  $\mu$ M). RNA was submitted to RT-PCR using gene-specific primers. The number of cycles was optimized for each primer pair. S19 was used as a control.

disturbing PLD (Fig. 8) and PLC (data not shown) pathways. An inhibitory effect of primary alcohols can be seen while no inhibitory effect of U73122 or edelfosine could be detected.

#### Responses of PLC, PLD, and DAGK Genes to the Various Treatments

The changes concerning the transcript levels of PLC, PLD, and DAGK genes (Supplemental Table VI) were extracted from the different comparisons of our microarray data. The nomenclature used for these genes are from Mueller-Roeber and Pical (2002), Qin and Wang (2002), and Gomez-Merino et al. (2004). For PLC genes, only *AtPLC2* showed a difference in transcript levels in one of our comparisons, with the transcript level at 22°C<sup>U73343</sup> being higher than at 22°C. For DAGK genes, only *AtDAGK7* showed a difference in transcript levels, with transcript abundance being higher at 4°C<sup>U73343</sup> compared to 4°C. For PLD genes, two genes, *AtPLD $\zeta$ 1* and *AtPLD $\zeta$ 2*, showed differences in transcript levels in a single comparison. Both genes had fewer transcripts at 22°C<sup>U73343</sup> than at 22°C and fewer transcripts at 22°C<sup>tertButOH</sup> when compared to 22°C. Besides, *AtPLD $\zeta$ 2* transcript levels were higher at 4°C than at 22°C as well as at 4°C with respect to 4°C<sup>tertButOH</sup>. This clearly reveals that under our conditions, PLC and DAGK genes were not induced by cold at a level higher than the cutoff designed for the microarray experiment. On the other hand, a single PLD gene (*PLD $\zeta$ 2*) was found to be cold induced.

## DISCUSSION

The fact that a temperature downshift induced the expression of genes known to be cold regulated in Arabidopsis plantlets in Arabidopsis cell suspensions

allowed us to consider this system as a good model for a genome-wide analysis of gene expression in response to a cold shock.

#### Analysis of Transcriptome Changes

The monitoring of cold-stress-induced changes within the transcriptome led us to identify 100 new cold-repressed and 324 new cold-induced genes. The fact that these genes had not been described in a similar microarray studies with the Arabidopsis Affymetrix GeneChip AtH1 (Vogel et al., 2004) could be due to differences in the model used: i.e. suspension cells versus plantlets. However, it could also reflect differences in the gene sets probed in the two arrays. All in all, the analyses of the genes either up-regulated or down-regulated (Supplemental Table I) by cold offer a good picture of the challenge faced by plant cells during a temperature downshift and an insight into the mechanisms activated to cope with this stress. For instance, after a 4-h exposure at 4°C, it can be seen that the first functional category of up-regulated genes is transcription, and it is also the first functional category of down-regulated genes. This indicates that, in response to cold, a complex pattern of gene induction is triggered that results in a succession of transcription waves. This will lead to new communication webs as shown by the induction, and also repression, of kinases, calcium-dependent kinases, protein phosphatases, or calcium binding proteins. In parallel to this, a total change in metabolism is triggered, with non-photosynthetic sources of energy being up-regulated. This correlates with metabolomic studies where the increased levels in organic acids of the citric acid cycle suggest an up-regulation of respiration (Cook et al., 2004; Kaplan et al., 2004). Conversely, photosynthesis is shut down, as shown by the repression of genes encoding photosystem II and chlorophyll *a/b*-binding proteins. This adjustment of light-harvesting antenna size is believed to be a photoprotective mechanism against the production of activated oxygen species. Indeed, the induction of pathways leading to the synthesis of flavonoids, carotenoids, or polyamines (Supplemental Table I) can be correlated with the cells experiencing a photooxidative stress because these classes of molecules have been implicated in photoprotection (Harvaux and Kloppstech, 2001; He et al., 2002). The fact that cells experience oxidative stress during a temperature downshift has been confirmed by the induction of genes encoding molecules involved in detoxification, such as lignols (Rice-Evans et al., 1997), ferritin, peroxidase, or  $\gamma$ -glutamylcysteine synthetase. Finally, our transcriptome analysis indicates that Arabidopsis cells in suspension can activate during a temperature downshift the synthesis of molecules that contribute to cold and to freezing tolerance, like amino acids, which can be seen as compatible solutes (genes involved in amino acid metabolism are indeed disturbed by a cold exposure; Supplemental Table I), or sugars, that can have a protective role for membranes

**Table V.** List of genes cold-regulated via PLC activity or PLD-produced PtdOH

Genes activated via both pathways are indicated. Genes are identified by their Arabidopsis Genome Initiative numbers. Forty-two genes are up-regulated via PLC activity, while 72 genes are up-regulated via the production of PtdOH by PLD. Sixteen genes are down-regulated via PLC activity, while 15 genes are down-regulated via the production of PtdOH by PLD.

Genes Up-Regulated by Cold		
via PLC Activity Only: 35	via PLC Activity and PLD-Produced PtdOH: 7	via PLD-Produced PtdOH Only: 65
At1g08230; At1g27930; At1g29150	At1g68550	At1g33600; At1g28370; At1g27710
At1g72150; At1g50480; At1g54270	At1g69295	At1g15100; At1g35140; At1g13190
At1g31970; At1g35560; At1g05420	At2g10920	At1g28360; At1g32920; At1g61890
At1g80310; At2g16480; At2g35940	At4g00940	At1g08650; At1g17090; At1g27730
At2g02870; At2g36970; At2g21660	At4g22330	At1g68500; At1g68360; At1g79110
At2g15880; At2g26280; At2g36950	At5g17860	At2g41380; At2g40140; At2g44500
At3g19320; At3g09870; At3g45010	At5g17460	At2g22430; At2g38480; At2g26530
At3g01470; At3g30390; At3g51660		At2g42530; At2g45680; At2g28350
At3g05220; At3g47380; At3g27010		At2g16900; At2g16900; At2g41410
At4g27520; At4g36760; At4g15530		At2g01420; At2g25060; At3g50970
At5g37260; At5g40450; At5g62350		At4g31840; At3g44450; At3g15210
At5g25340; At5g01010		At3g21560; At3g19553; At3g47600
		At3g47620; At3g54420; At3g10500
		At3g10930; At3g23170; At3g55790
		At4g36220; At4g36500; At4g39670
		At4g36010; At4g01950; At4g24960
		At4g27652; At4g02380; At4g20780
		At5g25340; At5g47370; At5g03210
		At4g33070; At5g04340; At5g49480
		At5g07440; At5g47230; At5g35735
		At5g41400; At5g42050; At5g64310
		At5g20790; At5g24600
Genes Down-Regulated by Cold		
via PLC Activity Only: 15	via PLC Activity and PLD-Produced PtdOH: 1	via PLD-Produced PtdOH Only: 14
At1g72430; At1g11140; At1g32410	At1g80920	At1g10150; At1g03610; At1g33055
At1g71030; At1g54740; At2g05000		At2g35230; At2g32150; At2g27830
At2g25200; At2g20670; At2g16140		At2g20670; At2g38820; At3g5207
At3g10610; At3g51910; At3g29240		At3g26510; At4g35770; At4g03510
At4g26400; At5g21170; At5g39785		At5g22920; At5g61590

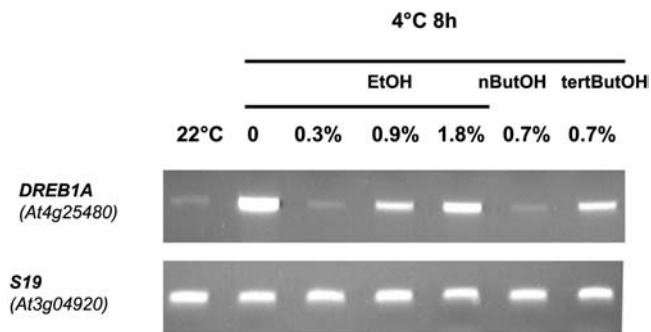
(Uemura et al., 2003). Proteins, like dehydrins of the cold-responsive/late embryogenesis abundant family, are also induced in our suspension cells. They are thought to protect membranes (Steponkus et al., 1998; Puhakainen et al., 2004). Finally, changes in lipid metabolism, such as induction of desaturases, can also contribute to the adaptive response of the cells by counteracting the cold-induced rigidification of membranes.

Since the changes in the transcriptome found under our conditions are in good agreement with the conclusions of previous transcriptomic and metabolomic studies, it was concluded that the cell suspension model could be used to investigate the role of the early activation of the PLC and PLD pathways in cold response.

#### PLD Activity Is an Element of Cold Transduction Leading to Gene Response

The PLD(s) activated during a temperature downshift can form phosphatidylethanol in the presence of ethanol, thus leading to a decrease in the amount of

PtdOH formed. This allowed for the determination of genes regulated by PLD-produced PtdOH by comparing data obtained after a cold stress applied in the presence or in the absence of this alcohol. However, it is important to note that ethanol does not inhibit PLD catalytic activity on phospholipids; therefore, the effect of ethanol on gene expression cannot be attributed to PLD activity but to PLD-produced PtdOH. Besides, the alcohol effect on cell metabolism is certainly not limited to an inhibition of the PLD pathway. Although primary alcohols can be substrates of transphosphatidylolation by PLD, they can also activate G proteins and fluidize membranes. On the other hand, secondary alcohols are not PLD substrates, but they can activate G proteins and fluidize membranes. While tertiary alcohols are not substrates of PLD, they cannot activate G proteins, but they can fluidize membranes. This raises the question of which alcohol should be chosen to serve as a control of the primary alcohol used. Comparing the effects of primary alcohols versus secondary alcohols on cold response could lead to



**Figure 8.** Effect of inhibitors of the PLD or PLC/DAGK pathways on the cold induction of the *DREB1A* gene. RNA was isolated from 5-d-old cell suspensions cultivated at 22°C with or without exposure for 8 h at 4°C. In the case of a cold exposure, when indicated, cell medium was supplemented with 0.3% (v/v), 0.9% (v/v), or 1.8% (v/v) ethanol or with 0.7% (v/v) tertButOH or with 0.7% (v/v) nButOH. RNA was submitted to RT-PCR using primers specific for *DREB1A*. *S19* was used as a control.

the removal of genes that are downstream of G protein activation. This is not the case when comparing the effects of primary alcohols versus tertiary alcohols. Nevertheless, since tertiary alcohols do not activate G proteins, the primary alcohol used had to have little effect on G protein, and ethanol has been reported to fulfill this condition (Kiss and Anderson, 1989; Munnik et al., 1995). Our control experiments show that neither 0.9% (v/v) ethanol nor 0.9% (v/v) tertButOH activate PLD activity, contrary to cold treatment. Therefore, we chose to compare the effect of ethanol and tertButOH on gene regulation by a cold shock. We showed that 87 genes, representing 94% of the genes that were cold regulated, displayed a transcript level difference between 4°C<sup>ethanol</sup> versus 4°C<sup>tertButOH</sup>, thus showing an inhibitory effect of the primary ethanol. As shown by  $\chi^2$  analysis, this proportion reflects a specific inhibitory action of ethanol versus tertButOH that can be considered as the inhibitory action on PLD-produced PtdOH and the cluster of 87 genes considered as driven by PLD-produced PtdOH. The dependence of some of these genes on the production of PtdOH by PLD during cold response was confirmed by northern-blot or RT-PCR analysis. We tested different concentrations of ethanol. For most genes, higher concentrations of ethanol led to an increase in transcript levels. This must be seen as a G protein effect. This does not necessarily mean that these genes are dependent on G proteins for their cold response but that G protein activation can activate their transcription. Interestingly, the cluster of genes driven by PLD-produced PtdOH included genes that were induced by the cold as well as genes that were down-regulated by this treatment. This indicates that PLD activation, leading to PtdOH production, triggers a signaling pathway that leads to the activation or repression of genes. The production of PtdOH by PLD has a positive action, since its inhibition mainly inhibits the cold response. This is an important fact indicating that PLD is implicated in the activation of the cold response and not in the turning off of this response.

As a control, we tested the effects of 0.9% (v/v) ethanol and 0.9% (v/v) tertButOH on gene expression at 22°C. Very few genes showed a difference in transcript levels after a 90-min incubation with these alcohols. This suggests a minor role, if any, of a basal PLD activity on gene regulation in nonstressed cells.

In the microarray experiment, a single PLD gene, *PLD $\zeta$ 2*, was found to be up-regulated by cold exposure. It would be interesting to elucidate whether or not this isoform is responsible for the early production of PtdOH by a PLD activity.

### PLC Activity Is an Element of Cold Transduction Leading to Gene Response

The identification of a cluster of genes that respond to cold via PLC was achieved by using U73122. This molecule acts on PLC, but we cannot rule out an action on other enzymes; therefore, its effect has to be compared to that of U73343, an analog with a significantly lower effect on PLC activity. When tested in response to a cold stress, increasing concentrations of U73122 led to a decreased production of PtdOH via the PLC/DAGK pathway, an effect that could not be reproduced with U73343. When RNA levels in the presence of U73122 during the cold shock were compared to RNA levels in the presence of U73343, we found an inhibition of the cold response genes in the presence of U73122. Indeed, 68 genes displayed a difference in transcript levels at 4°C in the presence of U73122 versus U73343, and 96% of these genes (65 genes) showed an inhibiting effect of U73422 on their cold response. Again  $\chi^2$  analyses confirmed a specific inhibitory action of U73122 versus U73343 on PLC. Therefore, the cluster of 65 genes can be considered as PLC driven. In order to know whether this regulation at 4°C by PLC activity was in a way correlated with a regulation already present at ambient temperature, microarray experiments were performed monitoring the effect of U73122 and U73343 on gene expression at 22°C. Of the 65 genes, seven of them showed a similar sensibility toward these molecules at 22°C as at 4°C. We therefore discarded these genes, thus leaving 58 genes identified as cold regulated via PLC activity.

Several of these genes were tested by northern and RT-PCR analyses and were confirmed to be PLC dependent. Here again, among these genes, some were up-regulated, while others were down-regulated by cold, indicating that PLC activation triggers a signaling pathway that can lead to both an activation and a repression of genes. However, with respect to cold, PLC appeared to have a positive action, since its inhibition led to an inhibition by the cold response, indicating that PLC is implicated in the activation of the cold response and not in the turning off of this response.

In the control experiments at 22°C, many genes, 1,507, showed a difference in transcript levels in the presence of U73122 versus U73343. Does this mean that a basal PLC activity exists that controls the expression of genes in nonstressed cells? This cannot

be ruled out. However, this might be an artifact due to the solvent (a mixture of DMSO and tertButOH) in which the U73122 and U73343 are dissolved. Indeed the effect of U73122 versus U73343 at 22°C might be effective not on the PLC-dependent basal expression of genes but on the expression regulated by DMSO (or tertButOH). This hypothesis is strengthened by the fact that 87% of the genes with transcript levels higher at 22°C<sup>U73122</sup> versus 4°C<sup>U73343</sup> also have lower transcript levels at 22°C<sup>U73343</sup> versus 22°C, suggesting that the effect at 22°C of U73122 versus U73343 mainly affects the gene expression disturbed by U73343 (certainly because of the solvents in which it is dissolved). Because at 4°C we did not consider the genes that showed a difference in transcript levels between 22°C<sup>U73122</sup> and 22°C<sup>U73343</sup>, we are certain that the effects we observe at 4°C are cold induced and not caused by a regulation via the solvents.

### Do PLC and PLD Activate the Same Transduction Pathways?

It is now obvious that the activation of PLC and PLD activities are elements in the transduction of the cold signal leading to cellular responses via downstream regulation of gene transcription. However, a remaining question is whether PLC and PLD activate two different pathways or if they coactivate a single pathway. Indeed, the same class of lipid molecules (PtdOH) is produced by both pathways: PLDs produce PtdOH, while PLCs produce InsP<sub>3</sub> and diacylglycerol, which can be phosphorylated to PtdOH. Cellular targets of PtdOH are beginning to be unraveled (Anthony et al., 2004; Testerink et al., 2004; Zhang et al., 2004; Testerink and Munnik, 2005). Interestingly, the molecular species of PtdOH produced by each pathway is different, since the PtdOH produced by PLC/DAGK is mainly 16:0/18:2 and 16:0/18:3 species, while the PtdOH produced by PLD is composed of these species and additional species like 18:3/18:2 and 18:2/18:2 (E. Ruelland and A. Zachowski, unpublished data). Our results indicate that each pathway mainly activates different targets. Indeed, among the 107 genes classified as up-regulated by cold either via PLC activity or via PLD-produced PtdOH, only seven were both PLC and PLD dependent; among the 30 genes classified down-regulated by cold via PLC or PLD, only one appeared to be dependent on PLC activity or on PLD-produced PtdOH. One explanation could be possible differences in the composition of the molecular species of PtdOH produced by each pathway, with specific molecular species activating specific targets. However, PLCs also produce diacylglycerol and InsP<sub>3</sub>, which may play a role in triggering distinct signaling cascades.

### Physiological Relevance of PLC and PLD Activating Two Distinct Pathways

In this work, we have shown that PLC and PLD activation participate in the cold response of Arabidop-

sis suspension cells. However, what is the link between PLC and PLD activation and the genetic regulons that have already been documented? The most documented regulon is CBF/DREB, although it does not account for all cold-responsive genes. Vogel et al. (2004) estimated that at least 70% of the cold-responsive genes, including transcription factors, were not affected by the expression of CBF transcription factors. This implies the existence of other genetic regulons. In their microarray analysis, Vogel et al. (2004) identified six genes encoding transcription factors that were coordinately regulated with *CBF2*. These genes, which can be expected to be primary transcription factors that should drive the cold induction of target genes, were *ZAT12* (At5g59820), *RAV1* (At1g13260), *MYB73* (At5g37260), *ZAT10* (At1g27730), *CZF1* (At2g40140), and *CZF2* (At5g04340). Interestingly, we show that the inhibition of PLC activity also inhibited the cold induction of *MYB73* and that the inhibition of the production of PtdOH by PLD also inhibited the induction of *DREB1A*, *ZAT10*, *CZF1*, and *CZF2*. This pleads for a major role of the PLC and PLD pathways in the cold response. However, concerning the link between PLD and the CBF regulon, it should be noted that although the cluster driven by PLD-produced PtdOH is enriched in CBF target genes, many identified CBF target genes were not found in this cluster. Furthermore, when an in silico promoter analysis was performed with PLD-driven genes, no overrepresentation of C-repeat elements could be detected (data not shown). This may indicate that PLD-produced PtdOH is going to induce a helper factor that is necessary for the action of CBF on some promoters but not on all promoters. Indeed, Maruyama et al. (2004) identified a conserved sequence (A/GCCGACNT) as a dehydration-responsive element core motif in direct downstream targets of *DREB1A* (*CBF3*). However, among the 13,434 Arabidopsis genes they monitored in their study, 531 contained the A/GCCGACNT motif. Many of these genes were not influenced by overexpression of *DREB1A*. This is an indication that this motif is a required cis-element for the expression of genes driven by *DREB1A* but that other elements, bound by unidentified factors, must be necessary for the expression of these genes. These helper factors are not necessarily the same for all CBF target genes, and one helper factor may be active in different genetic regulons. It is interesting to note that the overexpression of *ZAT10*, *MYB73*, or *CZF2* did not lead to the expression of cold-responsive genes (Vogel et al., 2004), which suggests that these genes encode such putative helper factors. We propose that PLD and PLC activation is upstream from the cold activation of such factors, *ZAT10* and *MYB73*, respectively.

## MATERIALS AND METHODS

### Materials

Edelfosine, U73122, and U73343 were from Calbiochem-Novabiochem. The culture medium for Arabidopsis (*Arabidopsis thaliana*) suspension cells

(Gamborg B5) was from Duchefa. [<sup>33</sup>P]-orthophosphate was purchased from Amersham Biosciences. Edelfosine (15 mM in water), U73122 (10 mM in DMSO:tertButOH, 27:53, v/v), and U73343 (10 mM in DMSO:tertButanol, 27:53, v/v) were used as stock solutions.

## Cell and Plant Cultures and Cold Treatment

The cell suspension was cultivated as described by Ruelland et al. (2002). For lipid analysis, the cold treatment of suspension cells was initiated by transferring the flasks to a water bath at 0°C, while maintaining orbital agitation. For RNA extraction, the cold treatment of suspension cells was initiated by transferring the flasks to a thermostated room at 4°C, under continuous illumination and orbital agitation. For plantlets, *Arabidopsis* ecotype Columbia were grown in a growth chamber at a temperature ranging between 22°C and 25°C. The light intensity was between 150 and 200 μmol m<sup>-2</sup> s<sup>-1</sup>. The photoperiod was 16 h light/8 h dark. Cold shock was performed by placing the plantlets into a thermostated room at 4°C under constant illumination.

## Lipid Analysis

In order to visualize PtdOH derived from DAGK activity, 6-d-old cells were labeled by incubation with 53 MBq L<sup>-1</sup> [<sup>33</sup>P]-orthophosphate for 2 h. In these conditions, visualized radioactive PtdOH mainly comes from the PLC pathway (Ruelland et al., 2002). On the contrary, in order to visualize PtdOH deriving from PLD activity, 5-d-old cells were labeled by incubation with 53 MBq L<sup>-1</sup> [<sup>33</sup>P]-orthophosphate for 24 h. An excess (3 mM) of nonradioactive orthophosphate was added 15 min before the cold shock to quench any remaining radioactive ATP. In these conditions, visualized radioactive PtdOH mainly comes from the PLD pathway (Ruelland et al., 2002). When required, pharmacological agents were added 15 min before the application of the cold shock. Lipids were extracted as described by Ruelland et al. (2002). Lipids were separated by thin-layer chromatography using an acidic solvent system (chloroform:methanol:acetone:acid acetic:water; 50:10:20:10:5, v/v). Radiolabeled phospholipids were detected by autoradiography, and radioactivity was quantified using a Storm PhosphorImager (Molecular Dynamics).

## RNA Extraction, RNA Hybridization, and RT-PCR

Total RNA from 500 mg to 2 g of suspension cells or plantlets was extracted according to Verwoerd et al. (1989). Ten or twenty micrograms of total RNA were fractionated on 1% agarose-formaldehyde gels and transferred onto Hybond N<sup>+</sup> membranes (Amersham). Hybridizations were carried out at 62°C using [<sup>32</sup>P]-dCTP-labeled probes generated using open reading frame cDNAs and random priming (Ready to Go; Amersham). Washes were carried out at 62°C, either with 2× SET, 0.2% SDS or 0.2× SET, 0.1% SDS. For RT-PCR, 1 μg of total RNA was treated with DNaseI (Sigma-Aldrich) according to the manufacturer's instructions, and first-strand cDNA was synthesized using the Omniscript reverse transcriptase kit (Qiagen). The reaction was carried out at 37°C for 1 h, stopped by heating to 70°C for 10 min, and immediately chilled on ice. One-tenth of the cDNA product was amplified in the Programmable-Thermal-Controller 100 (MJ Research) in a final volume of 25 μL of 1× PCR buffer, 200 mM each deoxynucleoside triphosphate, 1 mM each primer, and 2.5 units of Hot Start Taq polymerase (Qiagen).

## Transcriptome Studies

The microarray analysis was carried out at the Unité de Recherche en Génétique Végétale (Unité Mixte de Recherche, Institut National de la Recherche Agronomique 1165, Centre National de la Recherche Scientifique 8114) using the CATMA array (Crowe et al., 2003; Hilson et al., 2004), containing 24,576 gene-specific tags (GSTs) from *Arabidopsis* (Thareau et al., 2003). The spotting of the GST amplicons on array slides and the array analysis process have been described by Lurin et al. (2004). RNA was extracted from nonstressed cells or from treated cells. RNAs from three independent biological repetitions were pooled as recommended by Peng et al. (2003) in order to take into account the biological variation while minimizing the total cost. For each comparison, one technical replication with fluorochrome reversal was performed for each pool of RNA. RNA integrity was checked with the Bioanalyzer from Agilent. cRNAs were produced from 2 μg of total RNA from each sample with the Message Amp aRNA kit (Ambion). Then, 5 μg of cRNA was reverse transcribed in the presence of 300 units of SuperScript II (Invitrogen), cy3-dUTP, or cy5-

dUTP (NEN Life Science Products) for each slide. Samples were combined, purified, and concentrated with YM30 Microcon columns (Millipore). Slides were prehybridized for 1 h and hybridized overnight at 42°C in 25% formamide. Slides were washed in 2× SSC and 0.1% SDS for 4 min, 1× SSC for 4 min, 0.2× SSC for 4 min, and 0.05× SSC for 1 min and dried by centrifugation. Two hybridizations (one dye-swap) were performed. The arrays were scanned on a GenePix 4000 A scanner (Axon Instruments), and images were analyzed by GenePix Pro 3.0 (Axon Instruments).

## Statistical Analysis of Microarray Data

Experiments were designed with the statistics group of the Unité de Recherche en Génétique Végétale. The statistical analysis was based on one dye-swap (i.e. two arrays each containing 24,576 GSTs and 384 controls). The controls were used for assessing the quality of the hybridizations but were not included in the statistical tests or the graphical representation of the results shown in Results. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either log<sub>2</sub>(red/green) or log<sub>2</sub>(green/red) according to the experimental design. An array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features. Then, we performed a global intensity-dependent normalization using the loess procedure to correct the dye bias. Finally, for each block, the log ratio median calculated over the values for the entire block was subtracted from each individual log ratio value to correct print tip effects on each metablock. To determine differentially expressed genes, we performed a paired *t* test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extremes of variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method, which controls the FWER. We considered as being differentially expressed the genes with an FWER <5% (Lurin et al., 2004).

Sequence data from this article were deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) according to the MIAME standards (accession no. E-MEXP-259).

## ACKNOWLEDGMENTS

We thank M.L. Martin-Magniette (Unité de Recherche en Génétique Végétale, Evry, France) for help in experimental design and M. Hodges (Institut de Biotechnologie des Plantes, Unité Mixte de Recherche 8618, Université Paris-Sud, Orsay, France) for carefully reading the manuscript.

Received July 8, 2005; revised September 2, 2005; accepted September 7, 2005; published October 28, 2005.

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