The *Arabidopsis* Cold-Responsive Transcriptome and Its Regulation by ICE1 [W]

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To understand the gene network controlling tolerance to cold stress, we performed an *Arabidopsis thaliana* genome transcript expression profile using Affymetrix GeneChips that contain ~24,000 genes. We statistically determined 939 cold-regulated genes with 655 upregulated and 284 downregulated. A large number of early cold-responsive genes encode transcription factors that likely control late-responsive genes, suggesting a multitude of transcriptional cascades. In addition, many genes involved in chromatin level and posttranscriptional regulation were also cold regulated, suggesting their involvement in cold-responsive gene regulation. A number of genes important for the biosynthesis or signaling of plant hormones, such as abscisic acid, gibberellic acid, and auxin, are regulated by cold stress, which is of potential importance in coordinating cold tolerance with growth and development. We compared the cold-responsive transcriptomes of the wild type and *inducer of CBF expression 1* (*ice1*), a mutant defective in an upstream transcription factor required for chilling and freezing tolerance. The transcript levels of many cold-responsive genes were altered in the *ice1* mutant not only during cold stress but also before cold treatments. Our study provides a global picture of the *Arabidopsis* cold-responsive transcriptome and its control by ICE1 and will be valuable for understanding gene regulation under cold stress and the molecular mechanisms of cold tolerance.

INTRODUCTION

Low temperature is one of the major environmental stresses that many plants have to cope with during their life cycle. Plants from temperate regions have the capacity to cold acclimate, that is, to develop increased freezing tolerance after being exposed to low nonfreezing temperatures (Guy, 1990). Many physiological and molecular changes occur during cold acclimation (Thomashow, 1999). Among them, the transcriptional activation or repression of genes by low temperature is of primary importance (Thomashow, 1999). Early studies have identified a number of genes in plants that change expression under cold stress (Thomashow, 1994, 1999). A subset of the cold-responsive genes have in their promoters the dehydration-responsive element (DRE; 5′-TACCGA-CAT-3′)/C-repeat (CRT; 5′-TGGCCGAC-3′) with the common core motif (5′-CCGAC-3′). Transcriptional activators (DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR 1/C-REPEAT BINDING FACTOR [DREB1/CBF]) that are capable of binding

to DRE/CRT have been isolated from *Arabidopsis thaliana* using the yeast one-hybrid approach (Stockinger et al., 1997). Three members of the *CBF* gene family are rapidly and transiently induced by cold stress (Gilmour et al., 1998; Medina et al., 1999). Ectopic expression of *CBF1/3* (*DREB1B/A*) activated the expression of genes with the DRE/CRT promoter element at warm temperatures, which resulted in constitutive freezing tolerance (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998; Shinwari et al., 1998; Kasuga et al., 1999). Interestingly, *Arabidopsis* mutants with loss-of-function in *CBF2/DREB1C* show enhanced cold induction of *CBF/DREB1* target genes and increased freezing tolerance, suggesting a complex interplay among the *CBF/DREB1* family members and possibly also with other transcription factors (Novillo et al., 2004).

Recently, a constitutive transcription factor, INDUCER OF CBF EXPRESSION 1 (ICE1), which acts upstream of the CBFs in the cold-response pathway, was identified (Chinnusamy et al., 2003). ICE1 binds to the *CBF3* promoter and may activate *CBF3* expression upon cold treatment. The dominant *ice1* mutation blocks the cold induction of *CBF3* but not *CBF1* or *CBF2* (Chinnusamy et al., 2003).

The completion of the *Arabidopsis* genome sequence and technical advances in microarray analysis have allowed for the study of gene expression on a large scale. Several studies have used cDNA microarrays or Affymetrix GeneChips to identify cold-responsive genes in *Arabidopsis* (Seki et al., 2001, 2002; Fowler and Thomashow, 2002; Kreps et al., 2002). Expression profiling studies have also been performed with mutant plants or plants that overexpress a certain regulatory gene to understand the role of the genes in cold-responsive gene expression (Fowler

and Thomashow, 2002; Goda et al., 2002; Osakabe et al., 2002). However, the microarrays or GeneChips used in these studies contained no more than one-third of the *Arabidopsis* genome, and much of the *Arabidopsis* genome has not been statistically examined for transcript responses to cold stress.

In this study, we used the Affymetrix *Arabidopsis* 24K Gene-Chip representing ~24,000 genes to profile gene expression under cold stress. We identified 655 genes that are statistically cold upregulated and 284 genes that are downregulated. Our results suggest that cold stress triggers a multitude of transcriptional cascades because many of the early cold-responsive genes encode transcription factors that likely activate the genes induced late in the cold response. A number of genes important for the biosynthesis or signaling of plant hormones, such as abscisic acid, gibberellic acid, and auxin, are regulated by cold stress. The regulation of these genes might be important in coordinating cold tolerance with growth and development. We also determined the transcript profiles and their responses to cold stress in the *ice1* mutant. The *ice1* mutation affects the cold induction of a large number of genes, including many transcription factors. In addition, the *ice1* mutation alters the basal transcript levels of many cold-responsive genes. Our study provides a broad picture of the *Arabidopsis* cold-responsive transcriptome and its control by ICE1.

RESULTS AND DISCUSSION

Cold-Regulated Genes in *Arabidopsis*

We used the Affymetrix *Arabidopsis* ATH1 genome GeneChip, which contains >22,500 probe sets representing ~24,000 genes, to identify cold-regulated genes in *Arabidopsis*. Total RNA was prepared from *Arabidopsis* seedlings after 0, 3, 6, or 24 h cold treatment at 0°C. The 3- and 6-h time points were chosen to capture early responsive genes, and the 24-h point for late-responsive genes. A total of 100 to 150 seedlings from three plates was used to create pools of RNA used at each time point. As each plate can hold >300 plants, half of each plate contained the wild type and the other *ice1* seedlings. Thus, at each time point, the three plates produced one wild-type pool and one *ice1* pool of RNA. This procedure was repeated at each time point to produce a total of two biologically independent pools of RNA at each time point. The wild-type *Arabidopsis* used had a *CBF3* promoter-driven luciferase transgene, the background line of the *ice1* mutant (Chinnusamy et al., 2003).

To determine cold-regulated genes in *Arabidopsis*, we performed statistical analyses of the GeneChip data. Signal intensity data were first obtained by the Affymetrix Microarray Suite 5.0 program, where each cell signal intensity was background subtracted, weighted using one-step Tukey's biweight algorithm, averaged, and scaled to a globally normalized intensity of 500 (the manufacturer-suggested arbitrary value) for each chip. For statistical analysis, the signal intensity data were then analyzed with use of a two-stage linear statistical model and robust test statistics with the statistical package R (R Development Core Team, 2003; http://www.R-project.org) and the Limma package (Smyth, 2005). The 10,000 bootstrap simu-

lations (Efron and Tibshirani, 1993) were used to obtain non-parametric P values for testing a null hypothesis of no difference for gene-specific contrasts of expression levels between the different time points (see Methods for details). False discovery rates (FDRs) for various P value thresholds were later determined by the method of Benjamini and Yekutieli (2001) on the observed distribution of P values. Genes with <1% of FDR at any time point were considered significantly cold responsive. When this threshold was applied, 939 genes were determined to be cold regulated with 655 upregulated and 284 downregulated (Figure 1). Thus, ~3.9% of all *Arabidopsis* genes were determined to be cold responsive.

To validate the microarray data, we performed RNA gel blot analysis for three cold-regulated genes from our list. These included a protein phosphatase 2C (At3g11410), a small auxin upregulated RNA (*SAUR*) gene (At4g38840), and *TOUCH4* (At5g57560). The RNA gel blot results showed that the three genes are all cold regulated, and their expression kinetics from the RNA gel blots is very similar to those obtained from microarray analysis (Figure 2). These results support the validity of the *Arabidopsis* cold-regulated transcriptome from the GeneChip analysis.

Among the 655 cold upregulated genes, 128 were upregulated at 3 h, 195 at 6 h, and 581 at 24 h of cold treatment. Thus, most of the cold-upregulated genes are late-response genes (Figure 1). Indeed, Venn diagram analysis shows that 435 (66.4%) genes were induced exclusively at 24 h of cold treatment (Figure 1), while only 20 (3.1%) and 17 (2.6%) genes were upregulated exclusively at 3 or 6 h of cold treatment, respectively. Out of the cold upregulated genes, 66 genes (10.1%) had a high level of cold induction at all time points.

Most of the 284 downregulated genes were downregulated also only after 24 h of cold treatment. Only four
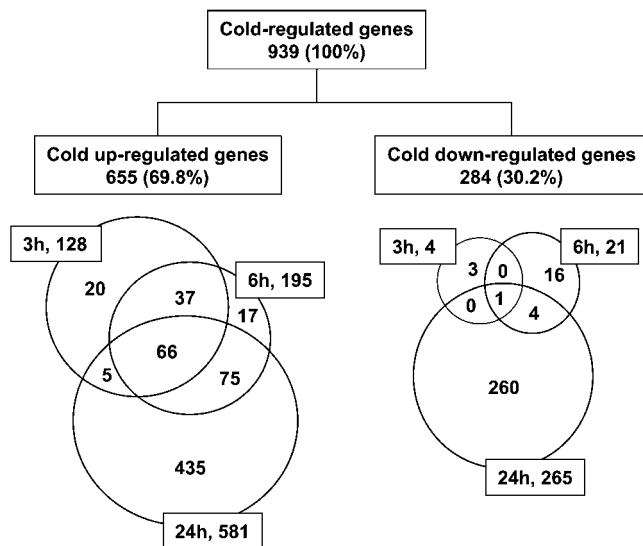

Figure 1. Venn Diagrams of Cold-Regulated Genes.

Percentages in parentheses were calculated with the total numbers of cold-regulated genes (939). Figures in rectangles indicate cold treatment hours (h) and total number of cold-regulated genes at each time point.
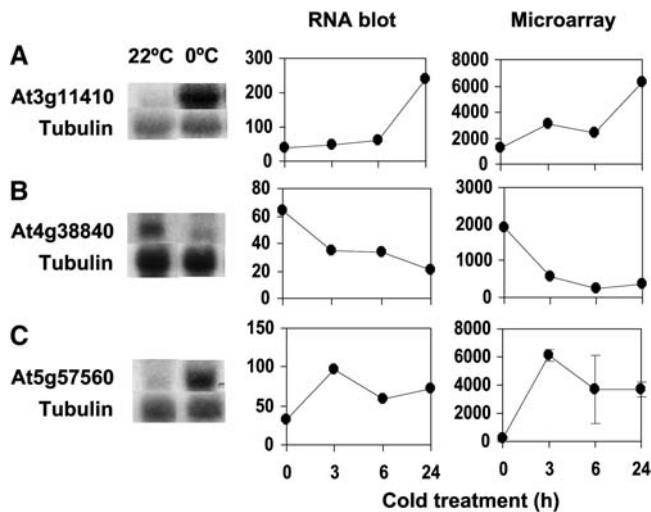
Figure 2. RNA Gel Blot Analysis of Cold-Regulated Genes Selected from Microarray Analysis.

Cold-responsive genes were tested by RNA gel blot analysis. For expression pattern comparisons, each corresponding gene transcript level was plotted using intensities from RNA gel blots and microarrays. The x axis shows time points during cold treatment (0°C), and y axis shows signal intensities in arbitrary units. Gene names and treatments for RNA gel blot pictures: **(A)** At3g11410 (protein phosphatase 2C, 0°C 24 h), **(B)** At4g38840 (*SAUR*, 0°C 6 h), and **(C)** At5g57560 (TCH4, 0°C 3 h). Tubulin was used as a loading control.

genes (At1g05610, glucose-1-phosphate adenylyltransferase; At2g23900, polygalacturonase; At5g34880, hypothetical protein; At5g38035, copia-like retrotransposon) showed a substantial downregulation at 3 h, and the downregulation of one of the four (At5g38035, copia-like retrotransposon) was also observed at 6 and 24 h (Figure 1). The contrasting results among the numbers of cold downregulated genes at each time point suggest that although cold downregulated genes may play a role in cold responses in *Arabidopsis*, the downregulation is not a major part of the early response to cold stress. The upregulation of early genes by low temperature may be important for both upregulation and downregulation of late-response genes.

Functional Categorization of Cold-Regulated Genes

To distinguish the kinetics of regulation under cold stress, we designated genes with transcript level changes only at 3 or 6 h or at both 3 and 6 h as early and transiently upregulated or downregulated, respectively. Genes that changed exclusively at 24 h were considered late upregulated/downregulated genes. Genes with expression changes at 3 and 24 h, 6 and 24 h, or 3, 6, and 24 h were grouped as early and continually upregulated or downregulated, respectively.

Our cold-regulated genes were categorized into 19 functional groups using the Functional Catalogue at http://mips.gsf.de/projects/funcat, with manual adjustment when necessary (Table 1; Ruepp et al., 2004). Interestingly, different functional categories appeared to be activated at different time points after cold

treatment. Although genes without significant similarity to known genes or functions were in fact the largest group of cold-regulated genes, our analysis here focused on genes with sequence homology to known genes. Among the early transiently upregulated genes, the largest group were genes involved in transcription and the next largest were signal transduction genes; 19 genes are involved in transcription, and 12 are in signal transduction (Table 1). On the other hand, out of the early continually upregulated genes, 26 and 22 are involved in transcription and cell defense, respectively (Table 1). Genes involved in transcription (68 genes) and metabolism (67 genes) were the major groups among late upregulated genes. The large number of genes involved in metabolism suggests that plants start to undergo substantial changes in metabolism later in the cold, after an initial inhibition of metabolism due to reduced enzymatic activities under cold. Previous physiological and metabolomic studies have shown many metabolic changes during cold acclimation, such as increased accumulation of soluble sugars and other compatible osmolytes (Wanner and Junttila, 1999; Cook et al., 2004). In summary, genes involved in transcription (113 genes) and metabolism (92 genes) are the two major groups of cold upregulated genes, which are followed by genes involved in defense (67 genes) and signal transduction (63 genes).

Cold downregulated genes showed slower kinetics. Only 20 genes were downregulated early and transiently, and five genes were downregulated early and continually under cold stress, while most of the genes (278 genes) were downregulated late by low temperature (Table 1). As the majority of cold downregulated genes were down after 24 h of cold treatment, late downregulated genes represent the major groups of cold downregulated genes. Among the late downregulated genes, genes involved in metabolism are the largest group (67), with fewer genes in other categories. Thus, low temperature stress mainly downregulates metabolism genes, and the downregulation happens late in the cold.

Cold Regulation of Signal Transduction Components

Consistent with a role of Ca^{2+} in early cold signal transduction, Ca^{2+} binding proteins were the main signaling components induced at the early time points during cold stress (Table 2; see Supplemental Tables 3, 4, and 10 online). In fact, two of the four early transiently upregulated protein kinases require indirect or direct Ca^{2+} involvement for kinase activity (At1g01140, SOS2-like protein kinase 6/CBL-interacting protein kinase 9; At5g66210, calcium-dependent protein kinase), making eight genes Ca^{2+} related out of 12 early transiently upregulated genes. Protein phosphorylation and dephosphorylation have been implicated in cold signal transduction. Indeed, the number of protein kinases and phosphatases with altered expression was the largest (total of 32 genes among 82 cold-regulated signaling genes) (Table 2; see Supplemental Table 10 online). The induction of protein kinases was observed to exhibit all types of kinetics, whereas protein phosphatase gene induction occurred preferably at the late time point (Table 2; see Supplemental Table 10 online). In addition, the only signaling component that was early and continually downregulated was a protein phosphatase 2C (At5g02760).

Table 1. Functional Categorization of Cold-Regulated Genes

Functional Category	Cold Upregulated								Cold Downregulated								Total	
	ETU		ECU		LU		Subtotal		ETD		ECD		LD		Subtotal			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Metabolism	6	7.5	19	12.0	67	13.7	92	12.6	2	10.0	1	20.0	67	24.1	70	23.1	162	15.7
Energy	1	1.3	5	3.2	18	3.7	24	3.3		0.0		0.0	6	2.2	6	2.0	30	2.9
Storage protein		0.0		0.0	1	0.2	1	0.1		0.0		0.0	0	0.0	0	0.0	1	0.1
Cell cycle and DNA processing		0.0		0.0	9	1.8	9	1.2		0.0	1	20.0	6	2.2	7	2.3	16	1.6
Transcription	19	23.8	26	16.5	68	13.9	113	15.5	1	5.0		0.0	19	6.8	20	6.6	133	12.9
Protein synthesis		0.0		0.0	4	0.8	4	0.5	2	10.0		0.0	2	0.7	4	1.3	8	0.8
Protein fate (folding, modification, and destination)	1	1.3	8	5.1	22	4.5	31	4.3	3	15.0	1	20.0	13	4.7	17	5.6	48	4.7
Protein with binding function or cofactor requirement		0.0	1	0.6	4	0.8	5	0.7		0.0		0.0	5	1.8	5	1.7	10	1.0
Cellular transport, transport facilitation, and transport routes	4	5.0	8	5.1	26	5.3	38	5.2		0.0		0.0	13	4.7	13	4.3	51	4.9
Cellular communication/signal transduction mechanism	12	15.0	11	7.0	40	8.2	63	8.7		0.0	1	20.0	18	6.5	19	6.3	82	8.0
Cell rescue, defense, and virulence	6	7.5	22	13.9	39	8.0	67	9.2		0.0		0.0	13	4.7	13	4.3	80	7.8
Interaction with the cellular environment		0.0	1	0.6		0.0	1	0.1		0.0		0.0		0.0	0	0.0	1	0.1
Interaction with the environment (systemic)		0.0	1	0.6	4	0.8	5	0.7		0.0		0.0	5	1.8	5	1.7	10	1.0
Transposable elements and viral and plasmid proteins		0.0	1	0.6	1	0.2	2	0.3	1	5.0	1	20.0	1	0.4	3	1.0	5	0.5
Cell fate		0.0		0.0	7	1.4	7	1.0		0.0		0.0		0.0	0	0.0	7	0.7
Development (systemic)		0.0	1	0.6	3	0.6	4	0.5		0.0		0.0	2	0.7	2	0.7	6	0.6
Biogenesis of cellular components	2	2.5	3	1.9	7	1.4	12	1.6	1	5.0		0.0	6	2.2	7	2.3	19	1.8
Subcellular localization	4	5.0	6	3.8	14	2.9	24	3.3		0.0		0.0	6	2.2	6	2.0	30	2.9
No clear classification/unclassified	25	31.3	45	28.5	156	31.8	226	31.0	10	50.0		0.0	96	34.5	106	35.0	332	32.2
Total	80	100.0	158	100.0	490	100.0	728	100.0	20	100.0	5	100.0	278	100.0	303	100.0	1031	100.0

Note that among 939 cold-responsive genes, 132 belong to multiple functional categories and 10 are missing in the Functional Catalogue. The missing 10 genes were manually grouped into different functional categories following database searches. ETU/D, early transiently upregulated/downregulated; ECU/D, early continually upregulated/downregulated; LU/D, late upregulated/downregulated.

Fourteen genes for receptor-like kinases (RLKs) were cold regulated (Table 2; see Supplemental Table 10 online). Notably, all these cold-regulated RLKs responded late in the cold, which suggests that they might perceive secondary signals generated by early cold responses. Among the cold-regulated RLKs, two RLKs have been studied in detail. The leucine-rich repeat transmembrane protein kinase, *RKL1* (At1g48480), was predominantly expressed in stomatal cells and was induced by wounding, pathogen, and salicylic acid (Tarutani et al., 2004a, 2004b). The *RECEPTOR-LIKE PROTEIN KINASE 1* (*RPK1*) gene (At1g69270) has been reported to be induced by abscisic acid (ABA), dehydration, high salt, and cold (Hong et al., 1997). Recent work indicates that *RPK1* plays an important role in ABA signal transduction (Osakabe et al., 2005). These results suggest that cold signal transduction shares at least in part some common pathways with other biotic, abiotic, and ABA signaling through these RLKs.

Intriguingly, the expression of a type-A response regulator, *ARR16* (At2g40670), and a type-B response regulator, *ARR10* (At4g31920), was reduced at the late time point during cold stress (Table 2; see Supplemental Table 10 online). These response regulators of the two-component system have been implicated in cytokinin signaling (Hwang and Sheen, 2001; Kiba

et al., 2002). Thus, this observation indicates an interaction between cytokinin signaling and cold response. Consistent with this notion, histidine kinase2 (At5g35750), another component of two-component systems, is also cold regulated (Table 2; see Supplemental Table 10 online).

The pseudoresponse regulator *APRR1* (At5g61380) was cold upregulated late, while another, *APRR5* (At5g24470), was upregulated early and continually. A third pseudoresponse regulator, *APRR2* (At4g18020), was cold downregulated late (Table 2; see Supplemental Table 10 online). Pseudoresponse regulators are proteins that lack the conserved Asp residue that in typical response regulators is the phosphorylation target of the upstream kinase in two-component systems (Hwang et al., 2002). *APRR1* is also called *TIMING OF CAB EXPRESSION1* and is an essential component of the central oscillator for circadian rhythms in *Arabidopsis* (Strayer et al., 2000; Mizuno, 2004). *APRR5* has also been implicated in circadian rhythms (Yamamoto et al., 2003; Fujimori et al., 2005). It is worth noting that our cold treatment started at noon, that is, 6 h after dawn, and the seedlings were grown in a 16-h-light and 8-h-dark cycle for 2 weeks before the treatments. A previous report showed that the peaks of mRNA accumulation of *APRR1* and *APRR5* occurred at 12 and 8 h after

Table 2. Cold-Regulated Signaling Genes

Category	Cold Upregulated								Cold Downregulated								Total	
	ETU		ECU		LU		Subtotal		ETD		ECD		LD		Subtotal			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Calcium binding protein	6	50.0	3	27.3	5	12.5	14	22.2		0.0		0.0	1	5.6	1	5.3	15	18.3
Protein phosphatase	1	8.3	1	9.1	8	20.0	10	15.9		0.0	1	100.0	1	5.6	2	10.5	12	14.6
Protein kinase	4	33.3	4	36.4	8	20.0	16	25.4		0.0		0.0	4	22.2	4	21.1	20	24.4
Receptor-like kinase		0.0		0.0	7	17.5	7	11.1		0.0		0.0	7	38.9	7	36.8	14	17.1
Blue light teceptor		0.0		0.0	1	2.5	1	1.6		0.0		0.0		0.0		0.0	1	1.2
Histidine kinase		0.0		0.0	0	0.0	0	0.0		0.0		0.0	1	5.6	1	5.3	1	1.2
Respone regulator		0.0	1	9.1	1	2.5	2	3.2		0.0		0.0	3	16.7	3	15.8	5	6.1
Lipid signaling protein	1	8.3	1	9.1	6	15.0	8	12.7		0.0		0.0	1	5.6	1	5.3	9	11.0
GTP-related protein		0.0	1	9.1	4	10.0	5	7.9		0.0		0.0	0	0.0	0	0.0	5	6.1
Total	12	100.0	11	100.0	40	100.0	63	100.0	0	0.0	1	100.0	18	100.0	19	100.0	82	100.0

Note that three pseudoresponse regulators (*APRR1*, At5g61380; *APRR2*, At4g18020; *APRR5*, At5g24470) are included in the response regulator category.

dawn with 16-h-light/8-h-dark photoperiod conditions (Matsushika et al., 2000). Therefore, our control (i.e., 0 h treatment at noon) expression level should already have been at least half or close to the maximum expression level. Significant cold induction of *APRR1* and *APRR5* genes took place mainly at 24 h (i.e., at noon after one full circadian cycle) under cold treatment. Thus, the higher expression levels of *APRR1* and *APRR5* genes at 0°C appear to be the consequence of cold regulation rather than circadian effects.

Nine genes involved in phospholipid signaling were cold regulated, and almost all (eight genes) were upregulated by cold stress (Table 2; see Supplemental Table 10 online). These include four phospholipase Cs (ATPLC1, At5g58670; ATPLC4, At5g58700; ATPLC5, At5g58690; a putative phospholipase C [PLC], At4g34920), one phospholipase D (PLDα1, At3g15730), two diacylglycerol kinases (ATDGK1, At5g07920; ATDGK2, At5g63770), one inositol polyphosphate 5-phosphatase (IP5-PII; At4g18010), and one 3'(2'),5'-bisphosphate nucleotidase/ inositol polyphosphate 1-phosphatase (IPPase; At5g63990) (see Supplemental Table 10 online). All of these genes were cold regulated at the late time point except for IP5PII (At4g18010) and ATDGK1 (At5g07920), which are early and transiently and early and continuously induced under cold stress, respectively (see Supplemental Table 10 online). Because all of these genes function in phospholipid-based signaling, their induction/repression by cold stress strongly suggests that phospholipid second messengers are an important part of cold signaling. PLCs act on phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ is known to trigger the release of Ca^{2+} from internal stores, and DAG has been shown to activate protein kinase C in animal cells (Irvine, 1992). There are no homologs of protein kinase C in *Arabidopsis*, and DAG is converted to phosphatidic acid (PA) by diacylglycerol kinases (Munnik, 2001). PA can also be generated by direct hydrolysis of membrane phospholipids, such as phosphatidyl-choline and phophatidylethanolamine by phospholipase D (PLD). There are accumulating lines of evidence that PA is involved in cold signaling. During cold stress, PA content increased in

Arabidopsis suspension cell cultures, and this increase was due to the activation of PLC and PLD (Ruelland et al., 2002). Studies with *Arabidopsis* mutant or overexpression lines of PLDs supported their involvement in plant cold tolerance (Welti et al., 2002; Li et al., 2004). Consistent with these reports, cold induction of genes involved in PA production, such as diacylgly-cerol kinases and a PLD, suggests that PA is an important part of plant cold responses as reported for wounding and osmotic stress (Frank et al., 2000; Wang et al., 2000; Katagiri et al., 2001).

The FRY1 bifunctional 3'(2'),5'-bisphosphate nucleotidase/ inositol polyphosphate 1-phophatase was shown to play an important role as an IPPase (Xiong et al., 2001). Loss-of-function mutations in *FRY1* resulted in a significant increase in the expression of cold-responsive genes, which supports a role of IPPase in attenuating the IP$_3$ signal triggered by cold (Xiong et al., 2001). The expression of one of its paralogs (Atg563990) located next to FRY1 on chromosome 5 was significantly increased during cold (see Supplemental Table 10 online), suggesting its role in cold stress as an IPPase. Because most of these IP$_3$-related genes were induced late, IP$_3$ signaling is likely to be a late event during cold stress. Nevertheless, the early and transient induction of IP5PII (At4g18010) suggests that an early involve-ment of IP$_3$ in cold signaling cannot be ruled out completely.

Cold stress also induced two Rab GTPase genes (*ATRABC1/ ATRAB18*, At1g43890; *ATRABG3d*, At1g52280) (Table 2; see Supplemental Table 10 online). Although no in planta function of these genes has been established in *Arabidopsis*, Rab GTPases are known to function in intracellular membrane trafficking (Zerial and McBride, 2001). Thus, cold upregulation of the two Rab GTPase indicates that active membrane trafficking might take place under cold stress.

A putative GDP/GTP pyrophosphokinase (RELA/SPOT HO-MOLOG 2 [RSH2], At3g14050) was early and continually upre-gulated by cold (Table 2; see Supplemental Table 10 online). This gene encodes a plant homolog of *RelA* and *SpoT* (van der Biezen et al., 2000). In bacteria, RelA (p)ppGpp synthase and SpoT (p)ppGpp hydrolase determine the level of guanosine tetraphos-phate (ppGpp) and guanosine pentaphosphate (pppGpp) in

response to starvation and other stress conditions. Accumulation of (p)ppGpp in response to unfavorable conditions initiates the stringent response. Among three *Arabidopsis RelA* and *SpoT* homologs, RSH1 interacts with a pathogen resistance protein (RPP5) in the yeast two-hybrid system and confers phenotypes associated with (p)ppGpp synthesis in *Escherichia coli* and *Streptomyces coelicolor* (van der Biezen et al., 2000). Recently, it was reported that RSH from the halophyte *Suaeda japonica*, a RelA and SpoT homolog more closely related to RSH2 and RSH3 than RSH1, confers salt tolerance when expressed in *E. coli* and *Saccharomyces cerevisiae* (Yamada et al., 2003). Thus, despite the lack of functional analysis in plants, the induction of *RSH2* under cold stress suggests a possible occurrence of (p)ppGpp-mediated signal transduction under cold stress.

Cold Regulation of Transcription Factors

Studies of the cold-induced *DREB1/CBF* family of transcription factors have demonstrated the importance of transcription factors in plant freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). To understand the regulation of transcription factors by cold, we surveyed their expression levels during cold stress (Tables 3 and 4; see Supplemental Table 11 online). Among the 655 cold upregulated genes, 113 (17.3%) genes are annotated to function in transcription. By contrast, only 20 transcription-related genes (7.0%) were found among the 284 downregulated genes (Table 3). Transcription category genes include both transcription factors that have DNA binding domain(s) and transcription activation/repression domain(s) and other factors that modulate the activity of transcription factors. There are 95 transcription factors and 18 other transcriptional regulators among cold upregulated transcription category genes (Tables 3 and 4; see Supplemental Table 11 online). Eighteen transcription factors and two other transcriptional regulators belong to the cold downregulated transcription category (Tables 3 and 4; see Supplemental Table 11 online).

When classified by their characteristic DNA binding domains, except for one that encodes a DNA binding protein with a non-classified binding domain (At3g61260), the 95 cold upregulated transcription factors fall into 15 families (Table 3). Transcription factors with APETALA 2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF) and Zn finger domains are the two major families, accounting for 21 genes and 27 genes of the 95 upregulated transcription factors, respectively. Interestingly, these two groups of transcription factors appear to act in different time frames. The AP2/ERF family was mainly induced early transiently and continually during cold, whereas the Zn finger family was mostly late induced under cold stress. Therefore, the AP2/ERF transcription factors may play a major role early in the cold response, as is also supported by the fact that DREBs/CBFs are in the AP2/ERF family and are important for the activation of many late cold-responsive genes (Fowler and Thomashow, 2002).

Our microarray data revealed that cold induction of basic leucine zipper (bZIP) transcription factors occurred mainly at a late time point (Table 3; see Supplemental Table 11 online). bZIP transcription factors play a role in plant pathogen responses, light signaling, and ABA and abiotic stress signaling (Jakoby et al., 2002). We found that two ABA-responsive element binding

Table 3. Cold-Regulated Genes Involved in Transcription: Transcription Factors with Known DNA Binding Domains

	Cold Upregulated								Cold Downregulated								Total	
	ETU		ECU		LU		Subtotal		ETD		ECD		LD		Subtotal			
Category	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
AP2/ERF	7	36.8	11	42.3	3	4.4	21	18.6		0.0		0.0	1	5.3	1	5.0	22	16.5
B3 (ARF)		0.0		0.0	1	1.5	1	0.9		0.0		0.0		0.0	0	0.0	1	0.8
bZIP		0.0	1	3.8	5	7.4	6	5.3		0.0		0.0		0.0	0	0.0	6	4.5
CCAAT		0.0		0.0	1	1.5	1	0.9		0.0		0.0		0.0	0	0.0	1	0.8
CG-1 (CAMTA4)		0.0		0.0	1	1.5	1	0.9		0.0		0.0		0.0	0	0.0	1	0.8
GRAS		0.0		0.0	2	2.9	2	1.8		0.0		0.0	1	5.3	1	5.0	3	2.3
Homeodomain		0.0		0.0	1	1.5	1	0.9		0.0		0.0	1	5.3	1	5.0	2	1.5
HSF	1	5.3	2	7.7	1	1.5	4	3.5		0.0		0.0		0.0	0	0.0	4	3.0
MADS		0.0		0.0		0.0	0	0.0		0.0		0.0	1	5.3	1	5.0	1	0.8
MYB	1	5.3		0.0	10	14.7	11	9.7		0.0		0.0	1	5.3	1	5.0	12	9.0
bHLH	1	5.3		0.0		0.0	1	0.9		0.0		0.0	7	36.8	7	35.0	8	6.0
NAC	1	5.3	1	3.8	7	10.3	9	8.0		0.0		0.0	1	5.3	1	5.0	10	7.5
SPB		0.0	1	3.8		0.0	1	0.9		0.0		0.0	1	5.3	1	5.0	2	1.5
Trihelix		0.0		0.0	1	1.5	1	0.9		0.0		0.0	2	10.5	2	10.0	3	2.3
WRKY	5	26.3	1	3.8	2	2.9	8	7.1		0.0		0.0		0.0	0	0.0	8	6.0
Zn	2	10.5	7	26.9	18	26.5	27	23.9		0.0		0.0	2	10.5	2	10.0	29	21.8
ETC	1	5.3	2	7.7	15	22.1	18	15.9	1	100.0		0.0	1	5.3	2	10.0	20	15.0
Total	19	100.0	26	100.0	68	100.0	113	100.0	1	100.0	0	0.0	19	100.0	20	100.0	133	100.0

Note that ETC includes the transcription regulatory proteins, one transcription factor with unclassified DNA binding domain (At3g61260), and general transcription factor TFIIB (At2g41630). AP2, APETALA2; ERF, ethylene-responsive element binding factor; ARF, auxin response factor; HSF, heat shock transcription factor; CAMTA, calmodulin binding transcription activators; CCAAT, CCAAT binding motif; SPB, squamosa promoter binding.

Table 4. Cold-Regulated Genes Involved in Transcription: Transcription Regulators Indirectly Involved in Transcription Processes

Category	AGI ID[a]	Gene Name	Kinetics
Chromatin	At1g63020	RNA polymerase IV (RNA polymerase D) subunit; NRPD1a	ECU
	At5g63330	DNA binding bromodomain-containing protein	ECU
	At5g50320	GCN5-related *N*-acetyltransferase (GNAT) family	LU
	At3g12980	Histone acetyltransferase 5 (HAC5)	LU
	At5g26040	Histone deacetylase, putative	LU
	At5g14270	Bromodomain-containing protein	LU
	At5g10550	Bromodomain protein like	ETD
Splicing	At1g28060	snRNP; similarity to U4/U6 small nuclear ribonucleoprotein hPrp3	LU
	At4g03430	Putative pre-mRNA splicing factor	LU
	At4g05410	U3 snoRNP-associated–like protein	LU
	At4g25500	STA1; Arg/Ser-rich splicing factor RSp40	LU
	At5g46920	Intron maturase, type II family protein	LU
Turnover	At3g44260	CCR4-associated factor 1-like protein	ETU
	At2g22090	UBP1 interacting protein, putative	LD
Other	At1g69250	Nuclear transport factor 2 (NTF2) family	LU
	At5g59950	RNA and export factor binding protein, putative	LU
	At2g42520	DEAD box RNA helicase, putative	LU
	At4g34910	DEAD/DEAH box helicase (RH16)	LU

[a] AGI, *Arabidopsis* Genome Initiative.

factors (*ABF1*, At1g49720; *ABF4/AREB2*, At3g19290) and *ABA INSENSITIVE 5* (*ABI5*, At2g36270) genes were cold induced. These *ABFs/ABREBs* are highly similar to *ABI5* and belong to group A bZIP transcription factors (Jakoby et al., 2002). Although they are all ABA responsive, the expression patterns of *ABFs/ABREBs* in response to various stress treatments are different (Choi et al., 2000; Uno et al., 2000). In addition, *Arabidopsis* plants overexpressing *ABF2/AREB1*, *ABF3*, or *ABF4/AREB2* display distinct stress responses (Kang et al., 2002; Kim et al., 2004), suggesting specific or partially overlapping functions of *ABFs/ABREBs* in stress responses.

Several transcription factors involved in plant development (e.g., ARF, GRAS, Homeodomain, MADS, and NAC) were regulated by cold stress (Table 3; see Supplemental Table 11 online). For example, auxin response factor 7/NONPHOTOTROPHIC

HYPOCOTYL 4 (*ARF7/NPH4*, At5g20730) was cold upregulated at the late time point. *ARF7/NPH4* functions in auxin-dependent differential cell expansion of hypocotyls (Stowe-Evans et al., 1998; Harper et al., 2000; Tatematsu et al., 2004; Okushima et al., 2005). Other examples include NAC transcription factors that are mainly cold induced at the late time point. Plant-specific NAC family transcription factors have a conserved NAC domain at the N termini of the proteins (Olsen et al., 2005) and have been implicated in plant development (Souer et al., 1996; Aida et al., 1997; Sablowski and Meyerowitz, 1998; Xie et al., 2000; Takada et al., 2001; Vroemen et al., 2003). It was recently reported that several NAC transcription factors are also involved in biotic and abiotic stress responses (Collinge and Boller, 2001; Hegedus et al., 2003; Fujita et al., 2004; Tran et al., 2004). Thus, these transcription factors might be effectors of cold signaling and function in reprogramming plant development to cope with cold stress.

Interestingly, four heat shock transcription factors (HSFs) and eight WRKY transcription factors were also cold responsive and were all upregulated (Table 3; see Supplemental Table 11 online). HSF and WRKY plant transcription factors have been implicated in heat stress and pathogen responses, respectively (Eulgem et al., 2000; Nover et al., 2001). These results suggest that several HSF and WRKY factors can be involved in plant responses to multiple stresses. Recently, it was shown that WRKY factors are important in senescence, morphogenesis, and ABA and gibberellin signaling in aleurone cells (Robatzek and Somssich, 2001; Johnson et al., 2002; Zhang et al., 2004; Xie et al., 2005). Also, WRKY expression was induced by various abiotic stresses (Pnueli et al., 2002; Rizhsky et al., 2002; Seki et al., 2002).

Most of the MYB transcription factors (10 out of 12 genes) were cold induced late, while most of the basic helix-loop-helix (bHLH) transcription factors (seven out of eight genes) were cold downregulated late (Table 3; see Supplemental Table 11 online). As MYB and bHLH proteins often interact with each other to control transcription (Stracke et al., 2001; Heim et al., 2003; Ramsay and Glover, 2005), this differential expression of MYB and bHLH transcription factors suggests that the regulation of some cold-responsive genes may be achieved by modulating the ratio of these partner factors.

Cold Regulation of Other Transcription Regulators

Our microarray data revealed that the expression levels of two mitochondrial genes (*cox1* and *nad6*) increased during cold stress (see Supplemental Table 2 online). *cox1* (AtMg01360) encodes cytochrome c oxidase subunit 1 and *nad6* (AtMg00270) encodes NADH dehydrogenase subunit 6. Related to this, we noticed that seven genes encoding a protein with a pentatricopeptide repeat (At1g74750, At3g57430, At3g60980, At4g36680, At5g02860, At5g25630, and At5g61370) are cold regulated (see Supplemental Tables 1, 5, and 9 online). All but At5g25630, which shows late downregulated kinetics, were late induced by cold. Proteins with a pentatricopeptide repeat are known to be involved in organellar RNA metabolism (Lurin et al., 2004). We reported previously that a mutation in nuclear-encoded mitochondrial complex I subunit resulted in impaired cold acclimation and reduced cold-responsive gene expression (B.-h. Lee et al., 2002), suggesting the importance of coordination of

nuclear cold-responsive gene expression and mitochondrial functions. Our microarray data further support the notion that proper organellar functions, especially mitochondrial electron transport, are important for plant cold responses, including cold-regulated gene expression.

Expression of many genes involved in RNA metabolism and chromatin remodeling was altered by cold stress (Table 4; see Supplemental Table 11 online). Expression of four pre-mRNA splicing factors (At1g28060, At4g03430, At4g05410, and At4g25500), two DEAD box RNA helicases (At2g42520 and At4g34910), and two RNA transport factors (At1g69250 and At5g59950) are increased by cold stress. These genes are known to be involved in RNA metabolism, including splicing, ribosome biogenesis, mRNA export, translational regulation, and RNA degradation (Farina and Singer, 2002; Jurica and Moore, 2003; Rocak and Linder, 2004). In addition, CCR4-associated factor 1 (CAF1)-like protein (At3g44260) is early and transiently cold induced. CAF1 is a critical component of the major cytoplasmic deadenylase in yeast, which initiates mRNA turnover by shortening of the poly(A) tail (Tucker et al., 2001). Also, UBP1-interacting proteins are thought to be involved in a complex that recognizes U-rich sequences in plant 3′ untranslated regions for the stabilization of mRNAs in the nucleus (Lambermon et al., 2002). Thus, induction of the CAF1-like protein and repression of the UPB1-interacting protein under cold stress might enhance the turnover of specific mRNA transcripts.

We have shown that mutations in an *Arabidopsis* DEAD box RNA helicase (*LOS4*, AT3G53110) affect mRNA export, cold sensitivity, and cold-responsive gene expression (Gong et al., 2002, 2005). Moreover, an *Arabidopsis sta1* mutant defective in one of our cold upregulated pre-mRNA splicing factors (At4g03430) displayed hypersensitivity to cold stress and missplicing of at least one cold-responsive gene, while it enhanced the transcript stability of some genes (B.-h. Lee and J.-K. Zhu, unpublished data). Taken together, the cold induction of several genes for RNA metabolism supports a strong connection between RNA metabolism and cold responses.

Interestingly, many genes that alter the chromatin status showed cold-regulated expression (Table 4; see Supplemental Table 11 online). One histone deacetylase (At5g26040), two histone acetylases (At3g12980 and At5g50320), and three bromodomain-containing proteins (At5g10550, At5g14270, and At5g63330) were among the cold-regulated genes involved in chromatin remodeling. Histone acetylases add acetyl groups to histone tails, whereas histone deacetylases remove acetyl groups from the histone tail. The bromodomain recognizes the acetylated Lys residues in histone tails (Eberharter and Becker, 2002). The kinetics of cold regulation of these genes differs, suggesting a complex and dynamic nature of cold-responsive gene regulation at the chromatin level.

We found that *NRPD1a* (At1g63020) was early and continually cold upregulated (Table 4; see Supplemental Table 11 online). *NRPD1a* encodes a plant-specific RNA polymerase IV subunit and is important in small interfering RNA-directed de novo DNA methylation important for silencing of transposon and other repetitive DNA in plants (Herr et al., 2005; Onodera et al., 2005). Recently, many small RNAs that are potentially associated with gene silencing were isolated from a small RNA library construc-

ted from abiotic stress–treated *Arabidopsis* (Sunkar and Zhu, 2004). Taken together, our finding suggests that some endogenous small RNAs might be increased by cold treatment, and *NRPD1a* may play an important role in this process.

In conclusion, cold-regulated gene expression is not only controlled by promoter binding transcription factors but may also be regulated at the posttranscriptional and chromatin levels.

Cold Regulation of Genes Related to Plant Hormone Biosynthesis and Signaling

Plant hormones are crucial regulators of growth and development. Plants grown under cold stress display growth and development patterns different from those under normal growth conditions, which might have to do with an altered hormone homeostasis and/or signal transduction in cold-stressed plants. To investigate this possibility, we inspected the expression pattern of hormone-related genes under cold treatment.

ABA is an important stress hormone that mediates abiotic stress signal transduction and tolerance. ABA accumulates in response to abiotic stress, such as drought and salt (Xiong and Zhu, 2003). Cold stress also increases endogenous ABA levels in plants but to a much lesser extent (Lang et al., 1994). Because ABA biosynthesis is mainly regulated at the transcriptional level (Xiong and Zhu, 2003), it is of interest to see whether ABA biosynthesis genes are regulated by cold. According to our microarray data, none of the known ABA synthesis genes were cold regulated, suggesting that ABA biosynthesis is not a major event in cold stress, consistent with previous studies (Lang et al., 1994).

Although ABA levels may not change greatly under cold stress, ABA signal transduction could be involved in cold responses. Cold stress induces many genes that are also induced by ABA and osmotic stress (Thomashow, 1999). Furthermore, many ABA-induced genes were induced by cold according to our microarray data (Table 5). As described above, a bZIP transcription factor, *ABF1* (At1g49720), which can bind to the ABA-responsive element in ABA-responsive promoters (Choi et al., 2000), is early and continually induced by cold (Table 5). A related gene, *ABF4/AREB2* (At3g19290), was also induced by cold. Thus, it is possible that the cold induction of some ABA-inducible genes might be mediated by ABF1 and ABF4.

Gibberellins (GAs) are involved in many plant developmental processes, including seed development, stem elongation, flowering, and fruit development (Richards et al., 2001). Many genes involved in GA biosynthesis have been isolated and characterized (Hedden and Kamiya, 1997). In several plants, such as rice (*Oryza sativa*), pea (*Pisum sativum*), and *Arabidopsis*, the transcript level of GA 20-oxidase, which catalyzes the conversion of C_{20}-GA into C_{19}-GA, is downregulated by GA. By contrast, treatment with GA biosynthesis inhibitor resulted in an increased level of GA 20-oxidase transcripts. In addition to this GA-induced negative feedback regulation of GA biosynthesis, positive regulation of a GA catabolic enzyme, GA 2-oxidase, was also reported (Thomas et al., 1999). Therefore, changes in the expression of these genes can indicate GA homeostasis under cold stress.

In this study, we found that two GA 20-oxidases (At3g60290 and At4g03060) were downregulated, and one, GA 2-oxidase

Table 5. Cold Upregulated Genes with Hormone-Related Roles

Hormone	AGI ID	Gene Name	Kinetics
ABA	At4g17615	Calcineurin B-like protein 1 (CBL1)	ETU
	At5g52310	Low-temperature-induced protein 78	ECU
	At1g20440	Dehydrin (COR47)	ECU
	At1g20450	Dehydrin (ERD10)	ECU
	At4g24960	ABA-induced–like protein	ECU
	At5g15960	Stress-induced protein KIN1	ECU
	At1g49720	ABA-responsive elements binding factor (ABF1)	ECU
	At2g36270	ABA-responsive element binding protein (ABI5)	LU
	At5g58670	Phosphoinositide-specific phospholipase C	LU
	At4g15910	Drought-induced protein (Di21)	LU
	At1g29395	Similar to the cold acclimation protein WCOR413 in wheat	LU
	At1g28200	GRAM domain-containing protein	LU
	At3g19290	ABA-responsive elements binding factor (ABF4/ABREB2)	LU
	At3g11410	Protein phosphatase 2C (PP2C), putative	LU
	At4g26080	Protein phosphatase ABI1	LU
	At5g50720	ABA-responsive protein (HVA22e)	LU
	At2g19450	Acyl CoA:diacylglycerol acyltransferase (DGAT)	LU
	At1g69270	Receptor protein kinase-related (RPK1)	LU
	At5g13200	GRAM domain-containing protein	LU
Auxin	At5g54490	PINOID binding protein containing putative calcium binding motifs	ETU
	At1g13260	AP2 domain transcription factor, putative (RAV1)	ETU
	At5g35735	Auxin-induced protein family	LU
	At5g20730	Auxin response factor (ARF7)	LU
	At5g53590	Auxin-induced (indole-3-acetic acid–induced) protein family	LU
Ethylene	At1g28370	Ethylene-responsive element binding factor 11, putative	ETU
	At4g17490	Ethylene-responsive element binding factor (ERF6)	ETU
	At5g51190	AP2 domain transcription factor, putative	ETU
	At4g11280	1-Aminocyclopropane-1-carboxylate synthase 6 (ACS6)	ECU
	At3g15210	Ethylene-responsive element binding factor 4 (ERF4)	ECU
	At5g47230	Ethylene-responsive element binding factor 5 (AtERF5)	ECU
	At1g67310	Calmodulin binding protein	LU
	At3g21420	Oxidoreductase, 2OG-Fe(II) oxygenase family	LU
	At4g20880	Ethylene-regulated transcript 2 (ERT2)	LU
GA	At1g02400	GA 2-oxidase, putative	LU
	At5g17490	GA response modulator, putative (RGL3)	LU

(At1g02400), was cold induced (Tables 5 and 6). These expression changes suggest the possibility of increased GA accumulation under cold stress. Although this potential GA accumulation under low temperature conditions might be part of the vernalization process that promotes flowering, it is also possible that GA might affect cold stress signaling and tolerance. Recently, it was demonstrated that in imbibed seeds, cold treatment enhances the biosynthesis of GA via the activation of *GA3Ox1* (or GA 3β hydroxylase, *GA4*, At1g15550) to promote seed germination (Yamauchi et al., 2004).

GA signaling is mediated through DELLA proteins that act as negative regulators (Olszewski et al., 2002). Upon GA perception, some DELLA protein is removed from the nucleus by degradation, releasing the repression of GA signaling. For example, in rice, the level of nuclear-localized SLR1-green fluorescent protein fusion decreased with GA treatment (Itoh et al., 2002). The *Arabidopsis* DELLA protein REPRESSOR OF GA (RGA) was shown to be regulated in a similar way (Silverstone et al., 2001). Rice has only one DELLA protein (Ikeda et al., 2001), but *Arabidopsis* has five: GA INSENSITIVE (GAI, At1g14920),

RGA (At2g01570), RGA-LIKE PROTEIN 1 (RGL1, At1g66350), RGL2 (At3g03450), and RGL3 (At5g17490). Not all of these proteins are regulated by degradation. GA treatment did not lead to GAI and RGL1 degradation (Fleck and Harberd, 2002; Wen and Chang, 2002). *RGL2* appears to be transcriptionally regulated by GA during seed germination (S. Lee et al., 2002). We found that *GAI* (At1g14920) was downregulated and *RGL3* (At5g17490) upregulated under cold, possibly by cold-induced GA (Tables 5 and 6). Thus, *GAI* and *RGL3* might be regulated at the mRNA level. Since the physiological function of RGL3 is unknown, our observation that it is upregulated by cold provides a clue to its function.

CONSTITUTIVE PHOTOMORPHOGENIC DWARF/DWARF3 (*CPD/DWF3*, At5g05690) was cold downregulated 24 h after cold treatment (Table 6). *CPD/DWF3* encodes a member of the cytochrome P450 90A family required for C23 hydroxylation of cathasterone to teasterone in brassinolide biosynthesis (Szekeres et al., 1996). *CPD/DWF3* expression is specifically downregulated by brassinolide treatment but not by other plant hormones (Mathur et al., 1998). Our observation of *CPD/DWF3* downregulation by

**Table 6.** Cold Downregulated Genes with Hormone-Related Roles

Hormone	AGI ID	Gene Name	Kinetics
Auxin	At2g45210	Putative auxin-regulated protein; *SAUR* gene family	ETD
	At4g38840	Auxin-induced protein-like; *SAUR* gene family	ETD
	At4g39950	CYP79B2	LD
	At4g31500	CYP83B1	LD
	At5g54510	Auxin-responsive–related protein	LD
	At3g25290	Auxin-induced protein family	LD
	At2g01420	Auxin transporter splice variant B (PIN4)	LD
	At1g29430	Auxin-induced protein, putative; *SAUR* gene family	LD
	At1g29500	Auxin-induced protein, putative; *SAUR* gene family	LD
	At4g15550	UDP-glucose:indole-3-acetate β-D-glucosyltransferase (iaglu)	LD
	At1g24100	Putative indole-3-acetate β-glucosyltransferase	LD
	At4g34770	Auxin-induced protein family; *SAUR* gene family	LD
	At1g72430	Auxin-induced protein-related; *SAUR* gene family	LD
	At1g28130	Auxin-regulated GH3 protein-related	LD
	At2g47750	Auxin-responsive protein-related	LD
	At2g36910	Multidrug resistance P-glycoprotein (pgp1)	LD
Brassinosteroid	At3g61460	BRH1 RING finger protein	ETD
	At5g05690	CPD, DWF3	LD
	At3g50750	Expressed protein	LD
Cytokinin	At5g35750	Histidine kinase-related protein; AHK2	LD
	At3g63440	Cytokinin oxidase family protein	LD
	At4g31920	ARR10; type B two-component response regulator protein	LD
	At2g40670	ARR16; type A two-component response regulator protein	LD
Ethylene	At5g25190	Ethylene-responsive element binding protein, putative	LD
GA	At3g60290	SRG1-like protein; GA 20-oxidase	LD
	At1g14920	GA response modulator (GAI/RGA2)	LD
	At4g03060	Putative oxidoreductase; GA 20-oxidase	LD
Jasmonate	At1g17990	12-Oxophytodienoate reductase, putative	LD
Salicylic acid	At1g21270	Wall-associated kinase 2 (WAK2)	LD

cold suggests that brassinosteroid level might be lowered in the cold, or cold signaling might interfere with brassinosteroid regulation of the *CPD/DWF3* gene. Consistent with this, expression of *BRASSINOSTEROID-RESPONSIVE RING-H2 1* (*BRH1*, At3g61460), whose transcript is downregulated by brassinolide (Molnar et al., 2002), was also downregulated at 6 h after cold stress.

We noticed that the expression of many genes with auxin-related roles was affected by cold stress. Twenty-one auxin-related genes showed cold upregulation or downregulation, and the majority of them (16 genes) were cold downregulated, while only five genes were cold upregulated (Tables 5 and 6). Among the 16 cold downregulated auxin genes, six belong to the *SAUR* gene family (Table 6). *SAUR* gene transcripts are very unstable (Hagen and Guilfoyle, 2002). Thus, cold stress may either prevent the stabilization of the *SAUR* gene transcripts (e.g., by reducing auxin levels) or decrease their transcription.

To examine the possibility of cold regulation of auxin levels, we monitored β-glucuronidase (GUS) activity in *Arabidopsis* seedlings expressing the *DR5-GUS* transgene. The *DR5-GUS Arabidopsis* plant has the GUS reporter gene driven by the synthetic auxin-responsive *DR5* promoter, which has been widely used as a reporter for endogenous auxin levels (Ulmasov et al., 1997). The GUS activity in *DR5-GUS* seedlings decreased after cold treatment (Figure 3), particularly in roots. This suggests that cold may decrease endogenous auxin levels, especially in roots, or in-

terfere with auxin sensitivity. Other cold-affected, auxin-related genes include those in signaling and transcription (At5g20730 and At5g54490), auxin conjugation/inactivation (At1g24100, At4g15550, At1g28130, At2g47750, and At5g54510), auxin biosynthesis (At4g31500 and At4g39950), and polar transport (At2g01420 and At2g36910).
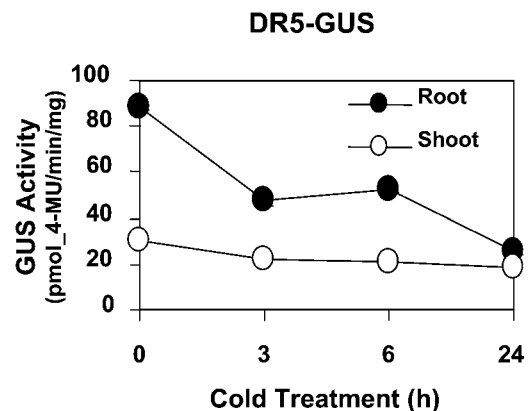
Figure 3. Quantification of *DR5-GUS* Activity during Cold Stress.

GUS quantification is shown in pmol·4-MU/min/mg on the *y* axis during cold treatment (h, *x* axis) of *DR5-GUS*–expressing *Arabidopsis*.

Obviously, cold is not the only factor that downregulates auxin-inducible genes. Wounding has a similar effect (Cheong et al., 2002). One proposed mechanism for downregulation by wounding is suppression of auxin signal transduction by a *NPK1*-like gene, a putative homolog of *ANP1* that negatively regulates auxin-responsive genes (Kovtun et al., 1998). In our microarray study, the *NPK1*-like gene (At2g30040) was early and transiently upregulated by cold, which suggests that cold repression of auxin-inducible genes might be mediated in a similar way as wounding (see Supplemental Table 2 online). Another proposed possibility of auxin-related gene downregulation by wounding was potentially lower levels of active auxin triggered by wounding (Cheong et al., 2002). This was based on the observation that the gene expression of a nitrilase, an auxin biosynthetic enzyme that catalyzes the conversion of indole-3-acetonitrile to indole-3-acetic acid (IAA), was downregulated and the transcript levels of two IAA glucosyltransferases, auxin conjugating enzymes, were upregulated by wounding (Cheong et al., 2002). In our microarray data, nitrilase genes were not significantly changed by cold, but IAA glucosyltransferase genes (At1g24100 and At4g15550) were downregulated. Therefore, although the disturbance of auxin homeostasis, transport, and signaling might all contribute to the downregulation of auxin-inducible genes by cold, the mechanisms appear to differ between the stresses.

Downregulation of auxin transport and auxin-responsive genes may eventually contribute to reduced plant growth rate under cold stress. Related to the growth regulation, we found that two members of the α-expansin gene family (Lee et al., 2001) were repressed at the late time point by cold (see Supplemental Table 9 online): expansin A1 (At1g69530) and A6 (At2g28950). Interestingly, three expansin-like genes (EXLA1, At3g45970; EXLA2, At4g38400; EXLA3, At3g45960) were cold upregulated (see Supplemental Table 2 online), which suggests that these expansin-like genes may have a distinct function in plant growth.

Cold-Regulated Gene Expression in the *ice1* Mutant

We have previously reported that the bHLH transcription factor ICE1 functions as an upstream regulator of *CBF* genes (Chinnusamy et al., 2003). The dominant *ice1* mutant shows reduced cold induction of *CBF3* and several *COR* (for cold responsive) genes that are under control of CBFs. To understand the role of ICE1 in global gene expression in the cold, the expression of the 939 cold-regulated genes in the wild type was
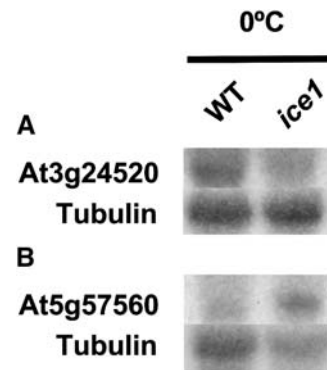

Figure 4. RNA Gel Blot Analysis of Cold-Regulated Genes in the Wild Type and *ice1*.

(A) At3g24520 (HSF1, 0°C 24 h).
(B) At5g57560 (TCH4, 0°C 0 h). Tubulin was used as a loading control.

compared with that in the *ice1* mutant at each time point during cold stress. As the *ice1* mutant was originally isolated due to its lower expression of *CBF3* (At4g25480) during cold stress, the *CBF3* expression level in both the wild type and *ice1* mutants was examined first. With the FDR of 1%, *CBF3* expression in *ice1* was statistically lower than the wild type only at 3 h after cold treatment. Using RNA gel blot analysis, we have previously observed that *CBF3* transcript levels were clearly lower in *ice1* than in the wild type at least until 24 h after cold treatment (Chinnusamy et al., 2003). Thus, the FDR of 1% appears to be too strict to be used as a threshold for the comparison of cold-responsive gene expression between the wild type and *ice1*. Another conventional FDR of 5% appeared to be an appropriate standard, as it rendered *CBF3* transcript levels to be significantly lower in *ice1* at 3, 6, and 24 h under cold stress. Therefore, 5% FDR was used here for the comparison of 939 cold-responsive genes between the wild type and *ice1*. The details of the statistical methods are described in Methods, and results from this analysis were confirmed by RNA hybridization with two randomly selected genes showing significantly different transcript levels between the wild type and *ice1* (Figure 4).

Among the 939 cold-regulated genes, the expression of 369 cold-regulated genes was affected in the *ice1* mutant (Table 7; see Supplemental Tables 12 and 13 online). Among them were 240 cold upregulated and 129 downregulated genes (Table 7;

Table 7. Comparison of Cold-Regulated Gene Expression between the Wild Type and *ice1*

Comparison Time Points Transcript Level in *ice1*	0 to 24 h			0 h			3 to 24 h (during Cold)		
	Higher	Lower	Subtotal	Higher	Lower	Subtotal	Higher	Lower	Subtotal
Cold upregulated (655)	142	101	240[a]	132	10	142	56	101	157
Cold downregulated (284)	72	58	129[b]	13	49	62	72	41	112[b]
Cold regulated (939)	214	159	369	145	59	204	128	142	269
Random (939)	64	56	120	41	30	71	50	44	94

[a] Three genes (At1g09070, At1g76590, and At4g15910) belong to both higher and lower categories at a certain time point.
[b] One gene (At4g26950) belongs to both the higher and lower categories at a certain time point.
Numbers in parentheses are total numbers of genes in each category.

Table 8. Cold-Regulated Transcription Genes Altered in *ice1*

Category	AGI ID	Kinetics	0 h	3 h	6 h	24 h	Gene Name
			Transcript Level in *ice1* at 0°C				
AP2	At1g28370	ETU	H				Ethylene-responsive element binding factor 11
	At1g77640	ETU	H			H	AP2 domain transcription factor, putative
	At5g51990	ETU		L	L		AP2 domain transcription factor-like protein
	At5g53290	ETU	H				AP2 domain transcription factor, putative
	At2g23340	ECU			L		AP2 domain transcription factor, putative
	At2g40350	ECU		H	H		AP2 domain transcription factor, putative (DREB2)
	At3g50260	ECU	H				AP2 domain transcription factor, putative
	At4g25480	ECU		L	L	L	DRE binding protein (DREB1A/CBF3)
	At5g07310	ECU	H				AP2 domain putative transcription factor
	At2g28550	LU			L		AP2 domain transcription factor RAP2.7
	At4g36900	LU				L	AP2 domain protein RAP2.10
	At4g36920	LU	H	H			Floral homeotic protein APETALA2
Myb	At5g67300	ETU	H				Myb-related protein, 33.3K
	At5g01200	LU			L	L	Myb family transcription factor
	At5g16560	LD			H		Myb family transcription factor
bHLH	At1g32640	ETU	H				bHLH protein (RAP-1); ATMYC2
	At2g23760	LU			L	L	BEL1-like homeobox 4 protein (BLH4)
	At1g09250	LD	L				bHLH protein; tRNA processing; chloroplast
	At1g18400	LD	L	L	L	L	Helix-loop-helix protein homolog–related
	At1g73830	LD	L	L			bHLH protein family
	At3g06120	LD		H		H	bHLH protein family
bZIP	At2g36270	LU	H	H			bZIP transcription factor AtbZip39
	At4g01120	LU			L	L	G-box binding bZip transcription factor GBF2/AtbZip54
	At4g34590	LU	H	H	H		bZIP transcription factor ATB2/Atbzip11
GRAS	At1g14920	LD	L	L		L	Signal response protein (GAI)
HSF	At3g24520	ECU		L	L	L	HSF1
MADS	At3g66656	LD	L				MADS box protein
NAC	At5g24590	ETU			L		No apical meristem (NAM) protein family
	At3g10500	LU	H		H		NAM family protein
	At2g33480	LD			H		NAM protein family
SPB	At1g76580	ECU			L	L	SPL1-Related3 protein
Trihelix	At5g01380	LU		H			Transcription factor GT-3a
WRKY	At2g38470	ETU	H				Putative WRKY-type DNA binding protein
	At2g46400	ETU	H				WRKY family transcription factor
	At4g01250	ETU	H			H	WRKY family transcription factor
	At4g31800	ETU	H	H			WRKY family transcription factor
	At2g30250	ECU	H				WRKY family transcription factor
	At1g62300	LU	H	H	H		Transcription factor WRKY6
Zn	At3g55980	ETU	H				Zn finger (CCCH-type) family protein
	At1g27730	ECU	H				Salt tolerance Zn finger protein; ZAT10
	At5g59820	ECU	H		H		Zn finger protein Zat12
	At1g10170	LU	H			H	NF-X1–type Zn finger family protein
	At1g25250	LU				L	Zn finger (C2H2 type) family protein
	At3g28210	LU	H	H	H	H	Zn finger protein (PMZ)–related
	At5g18550	LU	H				Zn finger–like protein
	At2g44380	LD				H	CHP-rich Zn finger protein, putative
Not classified	At3g61260	LU	L	L	L		Putative DNA binding protein
Chromatin	At1g63020	ECU	H				RNA polymerase IV (RNA polymerase D) subunit; NRPD1a
	At5g10550	ETD			H		Bromodomain protein-like
RNA Metab.	At4g03430	LU	H				Putative pre-mRNA splicing factor (STA1)
	At4g05410	LU	H	H			U3 snoRNP-associated–like protein
	At5g46920	LU			H		Intron maturase, type II family

H or L indicates that the transcript level is higher or lower, respectively, in *ice1* compared with the wild type. SPB, squamosa promoter binding; RNA Metab., RNA metabolism.

see Supplemental Tables 12 and 13 online). Thus, the expression of ~40% of cold-regulated genes was altered in the *ice1* mutant in comparison to the wild type. To see if the altered expression affected primarily cold-regulated genes, 939 non-cold-regulated genes were randomly selected, and the gene expression of the random 939 genes was analyzed. The analysis revealed that only the expression of 120 of these genes was significantly different between the wild type and *ice1*, with 64 being higher and 56 significantly lower in *ice1* (Table 7). In summary, the expression of many of cold-regulated genes was affected by the *ice1* mutation, and the *ice1* mutation has a differential effect on the expression of cold-regulated genes than on that of genes in general.

We then examined the basal level (i.e., at 0 h) of expression of cold-regulated genes in the wild type and *ice1*. Basal transcript levels of 204 of the 939 cold-regulated genes were affected in *ice1* (Table 7). Interestingly, among *ice1*-affected cold upregulated genes, a substantially larger number of genes had higher basal transcript levels in *ice1* than lower basal levels compared with the wild type (132 genes versus 10 genes). By contrast, *ice1*-affected cold downregulated genes had more genes with lower basal levels in *ice1* than higher basal levels relative to the wild type (49 versus 13) (Table 7). The results suggest that the wild-type ICE1 protein plays a critical role in maintaining the basal expression levels for both cold upregulated and downregulated genes.

A comparison of the expression levels of cold-regulated genes after cold treatment revealed that there are approximately twofold more cold upregulated genes in *ice1* with lower expression levels than those with higher expression levels (101 versus 56) (Table 7). By contrast, the number of cold downregulated genes with higher transcript levels in *ice1* was higher than that with lower expression levels (72 versus 41) (Table 7). These are opposite tendencies to those observed in the basal transcript levels in *ice1*. It suggests that ICE1 plays a large role in the cold activation of gene expression as well as in the downregulation of genes.

Because ICE1 is the most upstream transcription factor known in the cascades of gene expression under cold stress (Chinnusamy et al., 2003), we examined in more detail the effect of the *ice1* mutation on cold regulation of transcription factors (Tables 8 and 9; see Supplemental Table 11 online). Including *CBF3* (At4g25480), the expression of 52 (39.1%) out of the 133 cold-regulated genes involved in transcription was affected by the *ice1* mutation (Tables 8 and 9; see Supplemental Table 11 online). Before cold treatments (i.e., 0 h), expression levels of most cold-responsive transcription-related genes were higher in *ice1*, and all of them belong to cold upregulated genes (Table 8). Interestingly, the expression level of these genes, except for one Zn finger domain gene (At3g28210), became similar to the wild-type level as the duration of cold stress became longer. In other words, at 24 h after cold stress, these transcription-related genes with higher basal transcript levels in *ice1* did not show significant difference from the wild-type levels. This suggests that the responsiveness of cold induction (i.e., the rate of gene induction) and the basal levels of these genes are affected in *ice1*. Our results thus indicate that ICE1 regulates cold tolerance by controlling the basal expression and induction of many cold-responsive transcription factors.

Among the transcription-related genes with altered expression in *ice1*, AP2 domain transcription factors were the major group,

followed by bZIPs and WRKYs (Table 9). In particular, the expression of most of the cold-inducible WRKY factors (six out of eight) was enhanced in *ice1*, and the alteration mostly occurred at either 0 h or early time points (Tables 8 and 9). In fact, WRKY transcription factors and AP2 domain transcription factors were the major early cold-regulated transcription factors (Table 3). Thus, ICE1 seems to preferentially target early or upstream transcription factors in cold-regulated gene expression.

The *ice1* mutation also affected the expression of 31 (37.8%) of the 82 cold-regulated genes involved in signal transduction (Tables 10 and 11; see Supplemental Table 10 online). Genes encoding Ca^{2+}-signaling proteins, RLKs, and lipid-signaling proteins were the major genes whose expression was altered in *ice1* (Table 11). Expression of other cold-regulated protein kinases and protein phosphatase was largely not changed in *ice1*. The results suggest that the ICE1 pathway may involve lipid and RLK-mediated processes.

An examination of genes with plant hormone-related roles in *ice1* revealed that ABA- and auxin-related genes were predominantly affected in *ice1* (Tables 12 and 13). This indicates that the activation of ICE1 may be upstream of the hormone-related changes during cold regulation.

Recently, putative CBF3 and CBF2 target genes were identified using *Arabidopsis* transgenic lines overexpressing *CBF3* or *CBF2* (Maruyama et al., 2004; Vogel et al., 2005). We compared the reported CBF3- and CBF2-targeted cold-responsive genes with *ice1*-affected cold-responsive genes. Out of 102 genes identified as cold upregulated *CBF3* and *CBF2* target genes, 74 are in our cold upregulated gene list. These 74 genes include six *CBF3*-specific target genes, 18 genes targeted by both *CBF2*

Table 9. Numbers of Cold-Regulated Transcription Genes Altered in *ice1*

Category	Gene Number Altered in *ice1*
AP2/ERF (22)	12
B3 (1)	0
bZIP (6)	3
CCAAT (1)	0
CG-1/CAMTA4 (1)	0
GRAS (3)	1
Homeodomain (2)	1
HSF (4)	1
MADS (1)	1
MYB (12)	3
bHLH (8)	5
NAC (10)	3
SPB (2)	1
Trihelix (3)	1
Unclassified DNA domain (1)	1
WRKY (8)	6
Zn (29)	8
Chromatin remodeling (4)	2
RNA metabolism (6)	3
Total	52

Numbers in parentheses indicate the total number of cold-regulated genes in each category.

Table 10. Cold-Regulated Signaling Genes Altered in *ice1*

Category	AGI ID	Kinetics	Transcript Level in *ice1* at 0°C				Gene Name
			0 h	3 h	6 h	24 h	
Calcium binding	At2g43290	ETU				H	Putative calcium binding protein
	At5g37770	ETU	H				Calmodulin-related protein 2, Touch-induced (TCH2)
	At3g10300	ECU	H				Calcium binding EF-hand family protein
	At4g27280	ECU	H				Calcium binding EF-hand family protein
	At5g49480	ECU	H				Sodium-inducible calcium binding protein
	At3g51920	LU	H				Putative calmodulin
	At5g55990	LU			L		Calcineurin B-like protein 2 (gb|AAC26009.1)
	At4g16350	LD				H	Calcineurin B-like protein 6 (CBL6)
Protein phosphatase	At3g16800	LU			L		Protein phosphatase 2C (PP2C), putative
	At5g02760	ECD	L	L			Protein phosphatase-like protein
Protein kinase	At1g01140	ETU			L		SOS2-like protein kinase PKS6/CBL-interacting protein kinase 9 (CIPK9)
	At3g24550	ETU			L		Protein kinase-related
	At1g07150	ECU	H				Protein kinase family; MAPKKK13
	At3g57760	ECU	H				Protein kinase family protein
	At2g28930	LU		H			Protein kinase (APK1b)
RLK	At1g61380	LU	H				S-like receptor protein kinase
	At3g53810	LU	H	H	H	H	Receptor lectin kinase, putative
	At1g12460	LD			L	L	Leucine-rich repeat transmembrane protein kinase, putative
	At1g34210	LD				H	Somatic embryogenesis receptor-related kinase
	At1g68780	LD	L	L	L		Leucine-rich repeat protein family
	At3g23110	LD				H	Disease resistance family protein, contains leucine-rich repeat
	At4g30520	LD	L				RLK homolog
Histine kinase	At5g35750	LD	L		L	L	Histidine kinase (AHK2)
Response regulator	At4g18020	LD		H			Pseudoresponse regulator 2 (APRR2)
Lipid signaling	At4g18010	ETU	H				IP5PII
	At5g07920	ECU			L		Diacylglycerol kinase (ATDGK1)
	At5g58670	LU	H	H	H		Phosphoinositide-specific phospholipase C (ATPLC1)
	At5g58690	LU				L	Phosphoinositide-specific phospholipase C-line (MZN1.13)
	At5g58700	LU	H				Phosphoinositide-specific phospholipase C4 (PLC4)
	At5g63770	LU	H				Diacylglycerol kinase
GTP-related	At1g30960	LU	H				GTP binding protein, ERG-related

H or L indicates that the transcript level is higher or lower, respectively, in *ice1* compared with the wild type. Note that pseudoresponse regulators are included in the response regulator category.

and *CBF3*, and 50 *CBF2*-specific target genes. Four of the six *CBF3*-specific target genes (67%) were altered in *ice1*, whereas 19 of the 50 *CBF2*-specific target genes (38%) were affected by the *ice1* mutation (see Supplemental Tables 14 to 16 online). Fifteen of the eighteen genes (83%) targeted by both *CBF2* and *CBF3* showed altered expression in the *ice1* mutant (see Supplemental Tables 14 to 16 online). In addition, we found that only one (At4g02330) out of the 24 cold-responsive *ZAT12* target genes (Vogel et al., 2005) was affected by *ice1*. The results show that the *ice1* mutation has a more pronounced effect on the target genes of *CBFs*, particularly of *CBF3*, which is consistent with the observation that the dominant *ice1* mutation has a very strong effect on the cold induction of *CBF3*.

Conclusions

In this study, we identified 655 cold upregulated and 284 down-regulated genes in *Arabidopsis* using the Affymetrix *Arabidopsis* 24,000-gene GeneChip. Many transcription factors were induced during cold stress, particularly during early cold stress. By contrast, only one transcription factor was downregulated early

during cold stress. These results suggest that cold responses in plants are initiated mainly by transcriptional activation rather than repression of genes. The downregulation of other transcription factors later in the cold may be the result of early activation of transcription factors. In addition, a number of RNA metabolism

Table 11. Numbers of Cold-Regulated Signaling Genes Altered in *ice1*

Category	Gene Number Altered in *ice1*
Calcium binding protein (15)	8
Protein phosphatase (12)	2
Protein kinase (20)	5
RLK (14)	7
Blue light receptor (1)	0
Histidine kinase (1)	1
Response regulator (5)	1
Lipid-signaling protein (9)	6
GTP-related protein (5)	1
Total (82)	31

Numbers in parentheses indicate the total number of cold-regulated genes in each category.

Table 12. Cold Upregulated Plant Hormone-Related Genes Altered in *ice1*

Hormone	AGI ID	Kinetics					Gene Name

Hormone	AGI ID	Kinetics	Transcript Level Altered in *ice1*				Gene Name
			0 h	3 h	6 h	24 h	
ABA	At5g52310	ECU		L			Low-temperature-induced protein 78
	At1g20440	ECU		L			Dehydrin (COR47)
	At1g20450	ECU		L	L		Dehydrin (ERD10)
	At4g24960	ECU			L		ABA-induced–like protein
	At5g15960	ECU		L	L		Stress-induced protein KIN1
	At2g36270	LU	H	H			ABA-responsive element binding protein, putative
	At5g58670	LU	H	H	H		Phosphoinositide-specific phospholipase C
	At4g15910	LU	H			L	Drought-induced protein (Di21)
	At1g29395	LU			L		Similar to the cold acclimation protein WCOR413 in wheat
Auxin	At5g35735	LU	H				Auxin-induced protein family
Ethylene	At1g28370	ETU	H				Ethylene-responsive element binding factor 11, putative

H or L indicates that the transcript level is higher or lower, respectively, in *ice1* compared with the wild type.

genes and chromatin remodeling proteins were also cold regulated, suggesting their involvement in cold-responsive gene regulation.

Many metabolism-related genes were cold induced and some were cold repressed, consistent with dynamic changes in metabolism observed under cold stress (Cook et al., 2004). Another large number of cold-regulated genes are in the cell rescue, defense, and virulence category. Many of these are induced not only by cold but also by other abiotic and biotic stresses and by ABA. Different signal transduction pathways may crosstalk and converge in the activation of these stress genes.

One notable observation was the downregulation of many auxin-inducible *SAUR* genes by cold stress. These *SAUR* genes were also reported to be downregulated by wounding (Cheong et al., 2002). A database search at www.genevestigator.ethz.ch revealed that many *SAUR* genes are downregulated by many other stresses, including osmotic stress and heat stress (Zimmermann et al., 2004). Therefore, it seems that the downregulation of many *SAUR* genes is one of the general stress responses that may be responsible for altered plant growth and

development in response to stress. How are *SAUR* genes repressed by cold? According to our cold-regulated gene profiles, both auxin homeostasis and signaling appear to be disturbed by cold stress, as the expression of auxin polar transporter genes and one NPK1-like gene (At2g30040, see above) was changed in response to cold stress. Consistent with this, we found that *DR5-GUS* reporter activity was decreased in cold-treated *Arabidopsis*.

Under cold conditions, plants grow more slowly, and some even show growth defects or damage. Some of these cold-induced growth changes might be attributed to the slowing of photosynthesis and generally low metabolic activities in the cold (Kubien et al., 2003). Our microarray data revealed other potential causes of altered plant growth and development at low temperatures. We observed the downregulation of two expansin genes. Therefore, one possibility is that the downregulation causes reduced cell expansion, which in turn affects plant growth in the cold. In addition, the altered homeostasis of auxin and possibly other plant hormones such as ethylene, GA, and brassinosteroid might also perturb plant development at low temperatures. Related to this, some development-relevant transcription factors (e.g., ARF, GRAS, Homeodomain, MADS, and

Table 13. Cold Downregulated Plant Hormone-Related Genes Altered in *ice1*

Hormone	AGI ID	Kinetics	Transcript Level Altered in *ice1*				Gene Name
			0 h	3 h	6 h	24 h	
Auxin	At2g45210	ETD	H		H	H	Putative auxin-regulated protein; *SAUR* gene family
	At4g38840	ETD				L	Auxin-induced protein like; *SAUR* gene family
	At4g39950	LD	H		H	H	CYP79B2
	At4g31500	LD			H	H	CYP83B1
	At5g54510	LD				H	Auxin-responsive-related protein
	At3g25290	LD	H	H	H	H	Auxin-induced protein family
	At2g01420	LD	L	L	L	L	Auxin transporter splice variant B (PIN4)
	At1g29430	LD	L	L	L	L	Auxin-induced protein, putative; *SAUR* gene family
	At1g29500	LD	L	L	L		Auxin-induced protein, putative; *SAUR* gene family
Cytokinin	At5g35750	LD	L		L	L	Histidine kinase-related protein; AHK2
GA	At3g60290	LD	L	L			SRG1-like protein; GA 20-oxidase
	At1g14920	LD	L	L		L	GA response modulator (GAI/RGA2)

H or L indicates that the transcript level is higher or lower, respectively, in *ice1* compared with the wild type.

NAC) were cold responsive, suggesting their potential involvement in reprogramming plant development under cold stress.

Our comparison of cold-responsive gene expression profiles between the wild type and *ice1* mutant supports the important role of ICE1 in cold response gene regulation and cold tolerance in plants. ICE1 regulates the expression of many transcription factors, which in turn may activate or repress other downstream cold-responsive genes. ICE1 is a MYC-like transcription factor in the bHLH family (Chinnusamy et al., 2003). Our survey of MYC recognition sites in cold-responsive genes with altered expression in the *ice1* mutant did not reveal any significant differences in the occurrence of MYC binding sites between promoters from randomly chosen genes and the *ice1*-affected cold-responsive genes. This is probably because the consensus MYC recognition site (CANNTG) is not specific enough for computational detection, and the specific ICE1 binding site needs better definition.

Notwithstanding, the expression of many cold-responsive plant genes known to be critical in cold tolerance were affected in the *ice1* mutant. For example, AP2 domain transcription factors, as illustrated by the numerous studies on the *CBF* genes, were the major early cold-inducible transcription factors, and these were affected by *ice1*. In addition, two major cold-regulated genes related to ABA or auxin were altered significantly by the *ice1* mutation. These results show that ICE1 plays a major role in plant cold responses.

A previous study with an 8K Affymetrix GeneChip described 306 genes, consisting of 218 cold upregulated and 88 cold downregulated genes (Fowler and Thomashow, 2002). Comparison of cold-responsive genes between Fowler and Thomashow's data set and ours revealed that only 108 genes were in common (data not shown). This might be due in part to the different experimental conditions. Fowler and Thomashow (2002) grew plants in Gamborg's B-5 medium on phytoagar, whereas our plants were grown in Murashige and Skoog (MS) medium on regular agar with 3% sucrose, and we used 0°C for cold treatment, whereas Fowler and Thomashow (2002) used 4°C for cold stress. Another cause of this discrepancy is that the analyses in the other studies (Fowler and Thomashow, 2002; Kreps et al., 2002) had no control over the Type I error rate of their reported findings. Nevertheless, these results indicate that experimental and developmental conditions significantly affect gene expression profiles, even with similar stress treatments. Certainly, the genes commonly identified by microarray studies from different groups are the most robust cold-responsive genes.

In summary, we identified many new cold-regulated genes and determined the impact of the *ice1* mutation on global gene expression under cold stress. This information provides a foundation for further experiments to explore the network of gene regulation in the cold and to determine the function of cold-responsive genes in cold tolerance through mutant analysis, transgenic overexpression, and other molecular or cell biological approaches.

METHODS

Plant Materials

Bioluminescent *Arabidopsis thaliana* (Columbia *gl1*) with a *CBF3* promoter-driven luciferase transgene was used as the wild type for the microarray analysis because the *ice1* mutant was derived from the *CBF3-LUC* transgenic *Arabidopsis* (Chinnusamy et al., 2003). The wild-type and *ice1* mutant seeds were plated on MS agar plates supplemented with 3% sucrose. Seedlings were grown at 22°C with 16-h-light and 8-h-dark cycles for 2 weeks before being harvested. To avoid variations due to circadian rhythm, all cold treatments were started at 12 PM at 0°C under light and continued for 0 (untreated control), 3, 6, and 24 h.

Microarray Analysis

For Affymetrix GeneChip analysis, 20 μg of total RNA from the wild-type and *ice1* seedlings with (3, 6, and 24 h under light) or without cold treatment were extracted using the RNeasy plant mini kit (Qiagen), and the product was used to make biotin-labeled cRNA targets. The Affymetrix *Arabidopsis* ATH1 genome array GeneChip, which contains >22,500 probe sets representing ~24,000 genes, was used. Hybridization, washing, and staining were performed according to the manufacturer's instructions. Microarray data were extracted from scanned GeneChip images and analyzed by use of Microarray Suite version 5.0.1 (Affymetrix). When necessary, the data produced by Mircoarray Suite were exported into Microsoft Excel or Microsoft Access and further analyzed. For *cis*-element analysis, the GCG/SeqLab program in the WISCONSIN package version 10.3 was used.

GUS Assay

For GUS quantification, shoots and roots of 10-d-old DR5-GUS *Arabidopsis* after cold treatment (0, 3, 6, and 24 h) were extracted in 800 μL of assay solution (50 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% SDS, 10 mM β-mercaptoethanol, and 0.1% Triton X-100). After protein quantification by use of the Bradford method, 20 μg of total protein extract from each sample was added to 200 μL of 2 mM 4 methyl umbelliferyl-β-D-glucuronide (4-MU) in assay solution and incubated for 10, 20, and 30 min at 37°C. The reaction was stopped by adding 800 μL of 0.2 M Na_2CO_3. The enzyme activity was measured by use of Titertek Fluoroskan II (Flow Laboratories) and calculated in units of pmol·4-MU/ min/mg protein. The assay was performed three times. Cold treatment starting time was 12 PM as it was for the microarray.

Statistical Analysis

Data were analyzed with use of a two-stage linear statistical model and robust test statistics as described in the Limma package (Smyth, 2005) in the Bioconductor Suite of tools for the statistical package R (R Development Core Team, 2003; http://www.R-project.org). Global effects were first estimated by use of a standard linear model on the observed intensity values, fitting array, genotype, time, and genotype by time interaction as fixed effects in the analysis:

$$intensity_{ijklm} = \mu + array_i + genotype_j + time_k + genotype \times time_{jk} + residual_{ijklm},$$

where i indexes arrays, j indexes genotypes, k indexes time, l indexes oligos, and m indexes individual intensity values. Residuals from this model were then used in subsequent individual gene analyses. This procedure is automated in the Limma package (Smyth, 2005) found in the Bioconductor suite of tools (http://www.bioconductor.org). The Limma package creates robust individual gene t-statistics for each contrast of interest (i.e., oligo by genotype by time) constructed by use of a shrinkage estimator for individual gene variances. The Limma model fits individual gene means, array, genotype, time, and genotype by time interaction effects regressed on the global model-adjusted intensity values (i.e., the residuals from the initial linear model).

$$\text{residual}_{ijklm} = \mu_l + \text{genotype}_{jl} + \text{time}_{kl} + \text{genotype} \times \text{time}_{jkl}$$
$$+ \text{error}_{ijklm}$$

Parameters for the shrinkage estimator are obtained with use of an empirical Bayes method (for details, see Smyth, 2005).

Significance of the robust t-statistics was determined from P values obtained with 10,000 bootstrap simulations (Efron and Tibshirani, 1993), with a corresponding Limma analysis assuming complete exchangeability of the Limma model residuals. The Limma model residuals should contain only experimental error and thus represent values from the null hypothesis of no genotype, time, or genotype by time effects. The t-statistics from this procedure then empirically determine the distribution of the t-statistic under the null hypothesis. FDRs for various P value thresholds were later determined by use of the method of Benjamini and Yekutieli (2001) on the observed distribution of P values.

RNA Gel Blot Analysis

To validate the microarray data, biologically different RNA samples were prepared from the tissue treated as described. Twenty micrograms of total RNA was resolved in a standard RNA gel (1% agarose). RNA was then transferred onto nylon membranes (Stratagene). The RNA membranes were hybridized with appropriate probes after UV fixation. DNA fragments for probes were PCR amplified from either cDNA clones from ABRC or genomic DNA with the following primers: At3g24520, HSTF-24520F (5′-CGAGAAAGAACGGACAAAGC-3′) and HSTF-24520R (5′-AGCCACCTCGAAACAGAGAA-3′); At3g11410, PP2C-11410F (5′-GAT-TTGTTGCGGTGTTGTTG-3′) and PP2C-11410R (5′-CACTTCTCCGCAA-CATGAGA-3′); At5g57560, TCH4-57560F (5′-GAAACTCCGCAGGAACA-GTC-3′) and TCH4-57560R (5′-GCACCCATCTCATCCTTTGT-3′); At4g38840, IAAind-38840F (5′-AGATTCTCCGACAAGCCAAA-3′) and IAAind-38840R (5′-TCAGTTGAAGCGAGAAGCAA-3′). We used the LabWorks program version 4.0.0.8 (UVP Biolmaging Systems) for quantification of the RNA hybridization signal. Acquired band intensities were then normalized with use of respective tubulin band intensity.

Accession Number Data Deposition

Affymetrix data have been deposited in the GEO public repository under the accession number GSE3326.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Cold-Regulated Gene List.

Supplemental Table 2. Cold Upregulated Gene List.

Supplemental Table 3. Cold Early Transiently Upregulated (ETU) Gene List.

Supplemental Table 4. Cold Early Continually Upregulated (ECU) Gene List.

Supplemental Table 5. Cold Late Upregulated (LU) Gene List.

Supplemental Table 6. Cold Downregulated Gene List.

Supplemental Table 7. Cold Early Transiently Downregulated (ETD) Gene List.

Supplemental Table 8. Cold Early Continually Downregulated (ECD) Gene List.

Supplemental Table 9. Cold Late Downregulated (LD) Gene List.

Supplemental Table 10. Cold-Regulated Signaling Gene List.

Supplemental Table 11. Cold-Regulated Transcription Gene List.

Supplemental Table 12. Cold Upregulated Genes Whose Expression Is Affected by *ice1*.

Supplemental Table 13. Cold Downregulated Genes Whose Expression Is Affected by *ice1*.

Supplemental Table 14. Cold Upregulated CBF3-Specific Target Genes Whose Expression Is Affected by *ice1*.

Supplemental Table 15. Cold Upregulated CBF3 and CBF2 Target Genes Whose Expression Is Affected by *ice1*.

Supplemental Table 16. Cold Upregulated CBF2-Specific Target Genes Whose Expression Is Affected by *ice1*.

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