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ORIGINAL RESEARCH

Using Specialized cDNA Microarrays to Analyze *Arabidopsis* Gene Expression Under Cold Stress

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Abstract Cold acclimation enables plants to withstand low but non-freezing temperatures. Biochemical and physiological changes include a reduction in tissue water content and altered composition of membrane lipids. These responses are correlated with fluctuations in the expression of cold-induced genes such as *LTI* (low-temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation), and *ERD* (early dehydration-inducible). We performed time-course experiments with specialized cDNA microarrays comprising 712 cDNAs selected by SAGE and SSH methods. Expression dynamics were monitored in the leaves of *Arabidopsis*. Profiles of nine samples from plants chilled for various time periods revealed 264 cold-inducible genes and 33 repressed genes, for which expression was altered (up or down) by at least twofold. These included not only several previously reported cold-regulated genes, e.g., *rd*, *lea*, and CBF3, but also candidate genes such as those for alcohol dehydrogenase and transport inhibitor response 1. All genes were grouped according to their expression patterns; most tended to shift their expression at 3~8 h after cold treatment. Two cold-associated transcriptional activators, CBF2 and CBF3, did not have parallel patterns of expression, although both were induced within 15 min. Our results suggest different roles for CBF2 and CBF3 in the signal-transduction pathway for cold acclimation. We believe that, compared with standard differential screening, our microarray analysis is a more useful technique for the selection of new candidate genes responsible for cold acclimation.

Keywords Specialized cDNA microarrays · *Arabidopsis* · Gene expression · Cold stress

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Temperature is one of the most important environmental factors influencing physiology, growth, and development. Many plants utilize mechanisms of cold acclimation (Levitt 1980) to withstand periods of low but non-freezing temperatures. Guy et al. (1985) have established that changes in gene expression occur with cold acclimation, a conclusion that has prompted numerous investigations to identify and characterize cold-responsive genes (Thomashow 1999). *Arabidopsis* is now used as a model plant in such research involving new molecular and genetic technologies.

Cold acclimation involves several biochemical and physiological changes, most notably a decline or cessation of growth; reduced tissue water content (Levitt 1980); a transient increase in abscisic acid (ABA) levels (Chen et al. 1983); altered membrane lipid composition (Lynch and Steponkus 1987; Uemura et al. 1995); the accumulation of compatible osmolytes, e.g., proline, betaine, polyols, and soluble sugars; and higher levels of antioxidants (Nomura et al. 1991; Koster and Lynch 1992; Kishitani et al. 1994; Murelli et al. 1995).

Plant cell membrane systems are the predominant site of freezing injury (Steponkus 1984), with such damage associated primarily with severe dehydration (Steponkus et al. 1993). Thus, a key function of cold acclimation is to

stabilize those membranes via multiple mechanisms. For example, alterations in membrane lipid composition are correlated with membrane cryostability (Steponkus 1984; Uemura and Steponkus 1994; Uemura et al. 1995). Moreover, the accumulation of sucrose and other simple sugars that typically occurs during cold acclimation also seems likely to contribute to membrane stabilization, as demonstrated in vivo (Strauss and Hauser 1986). Certain novel hydrophilic and LEA (late embryogenesis abundant) polypeptides also participate in the stabilization of membranes against freezing injury (Welin et al. 1994).

Many of these responses are regulated through changes in gene expression (Thomashow 1998). Numerous cold-induced genes have been cloned from a variety of plant species by differential screening of cDNA libraries and other techniques. These COR genes include *LTI* (low-temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation), and *ERD* (early dehydration-inducible). Others encode proteins with known enzymatic functions, such as alcohol dehydrogenase (Jarillo et al. 1993), phenylalanine ammonia lyase, chalcone synthase (Leyva et al. 1995), fatty acid desaturase (Gibson et al. 1994), lipid transfer protein (Hughes and Pearce 1988), a translation initiation factor (Dunn et al. 1993), thiol protease (Shaffer and Fischer 1988), catalases (Prasad et al. 1994), and delta-pyrroline-5-carboxylate synthase (Yoshiba et al. 1995). Some show similarity to a group of proteins involved in dehydration, including dehydrin- or LEA-like proteins (Gilmour et al. 1992), antifreeze proteins (Kurkela and Franck 1990), heat shock proteins, or molecular chaperones (Anderson et al. 1994a, b; Ukaji et al. 1999).

Although the underlying mechanisms by which plant cells perceive and transduce a cold signal to activate these cellular responses are unclear, evidence is mounting that calcium is an important second messenger involved in activating the cold acclimation response (Monroy and Dhindsa 1995; Knight et al. 1996; Sheen 1996). The phytohormone abscisic acid (ABA) also plays a key role (Chen et al. 1983; Heino et al. 1990; Gilmour and Thomashow 1991; Lang et al. 1994). Nevertheless, *COR* genes can also be induced by low temperature in both ABA-deficient and ABA-insensitive mutants (Gilmour and Thomashow 1991; Nordin et al. 1991), thereby indicating that some low-temperature signaling pathways act independent of ABA. C-repeat-binding factors (CBF) and dehydration-responsive element-binding protein 1 (DREB1) have been identified as transcription activators for COR-gene expression (Jaglo-Ottosen et al. 1998; Liu et al. 1998). For example, ectopic expression of CBF genes confers tolerance against freezing and other related stresses in plants (Jaglo-Ottosen et al. 1998; Liu et al. 1998).

Gene expression profiling using cDNA macroarrays (Welin et al. 1994; Seki et al. 2002a) or microarrays (Sheen 1996) is a novel approach for identifying even more transcripts and pathways related to tolerance mechanisms. Abiotic-stress transcriptome profiling in model species such as *Arabidopsis* has revealed several new stress-related pathways in addition to the previously well-described CBF pathway (Sheen 1996; Kreps et al. 2002; Seki et al. 2002a). Therefore, to better understand the processes involved in cold acclimation and to find new candidates with significant roles in freezing tolerance, we applied the DNA microarray technique to monitor relative abundances of transcripts and expression patterns by cold-related genes in *Arabidopsis* leaves, as well as to examine genes not previously thought to be regulated during that process of acclimation.

Materials and Methods

Plant Materials and Growing Conditions

Plants of *Arabidopsis thaliana* cv. Columbia were grown for 3 weeks in an environmentally controlled chamber at 23°C under constant light (approximately 100 μmol m^{-2} s^{-1}). Cold treatment (exposure to 0°C) was then applied for 0, 0.25, 0.5, 1, 3, 8, 16, 24, or 72 h. Those selected durations were based on our preliminary study and several other reported results (Welin et al. 1994; Gilmour et al. 1998; Xin and Browse 2000; Thomashow 2001). Immediately after each test period, leaves were harvested and stored at −80°C.

cDNA Microarrays

cDNA microarrays were prepared with 712 selected cDNA probes based on SAGE and SSH data from our laboratory. Probes included those for genes either induced or repressed under cold-stress conditions, as well as those for several known housekeeping genes. The duplicated spots were arranged in 16 subgrids (10 rows and columns) for a total of 1,424 elements per slide.

Preparation of RNAs and Labeled Targets

Frozen leaf samples were homogenized in liquid nitrogen and total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was reverse-transcribed in the presence of Cy3- or Cy5-conjugated dUTP (Perkin Elmer Life Sciences, MA, USA) and labeled cDNA was synthesized. Hybridization was performed according to the Brown protocol (http://cmgm.stanford.edu/pbrown), with minor modifications. Briefly, 100 μg of total RNA prepared from control (0 h non-treated) or cold-treated *Arabidopsis* leaves was mixed with 4 μg of oligo-dT primers (5′-

TTTTTTTTTTTTTTTTTTTT(A/C/G)(A/C/G/T)-3′) in 15.4 μl of water. Samples were heated to 65°C for 10 min, then cooled on ice. To this mixture, the following components were added to obtain a reaction mixture in a 30-μl total volume: 1× Superscript II reverse transcriptase buffer (Life Technologies, UK); 0.01 M DTT; 0.5 mM each of dATP, dCTP, and dGTP; 0.2 mM dTTT; 3 nmol of either Cy3-DUTP or Cy5-DUTP; and 2 μl of Superscript II reverse transcriptase. After incubation at 42°C for 2 h, the unincorporated nucleotides were removed through QIAquick columns (Qiagen). Each column was eluted twice with 80 μl of ddH_2O, and the two reaction products (one with Cy3 labeling, the other with Cy5 labeling) were combined. Afterward, 20 μl of 1 μg μl^{-1} human Cot-1 DNA (Life Technologies), 2 μl of 10 μg μl^{-1} poly A RNA (Sigma, MO, USA), and 2 μl of 10 μg μl^{-1} yeast tRNA (Life Technologies) were added and the samples were placed in a Microcon-30 filter (Millipore, MA, USA). The remaining volume of the filter cartridge was filled to the upper line with ddH_2O and centrifuged at 14,000×*g* for 12 min. The labeled cDNA was collected by placing the sample reservoir upside down on a new collection tube that was spun for 30 s. Afterward, 10 μl of 20× SSC, 20 μl of formamide, and 2 μl of 2% SSC were added to 8 μl of labeled cDNA. This was then denatured by placing it in a 100°C water bath for 3 min before centrifuging at 14,000×*g* for 2 min.

Hybridization and Washing Conditions for cDNA Microarrays

Labeled and denatured cDNA samples were spread on microarray slides that were covered with slips (VWR Scientific, PA, USA) for hybridization. Five drops (~50 μl) of 5× SSC were placed on a separate part of the well in a GT-Hyb-chamber II™ (GenomicTree, Korea) to provide humidity. The hybridization chamber was submerged in a 42°C water bath for 16 h. Afterward, the slides were removed and placed in a GT Washing Rack™ (GenomicTree) submerged in pre-warmed (42°C) washing solution 1 (1× SSC, 0.2% SDS) with the array faces of the slides tilted down. After the cover slips slid off, the slide rack was plunged up and down three times and the chamber was stirred gently for 4 min. The rack was carefully transferred to washing solution 2 (0.1× SSC, 0.2% SDS) and cleansed for 4 min with gentle stirring, then placed in solution 3 (0.1× SSC) where it was washed twice for 2.5 min each. Following the last wash, the slides were immediately dried by centrifugation for 5 min at 600 rpm.

Data Acquisition and Analysis of cDNA Microarrays

Washed slides were immediately scanned under an ArrayWoRx (Applied Precision, Issaquah, WA, USA). To maximize the camera's dynamic range without saturation and to normalize the signal intensities of two channels, we adjusted the exposure setting so that the intensity level for the brightest spot on a slide was 80~90% and the signal ratio of the β-tubulin and actin-2 (internal control) was approximately 1.0. Intensity values were quantified from the resultant pairs of TIFF files using ImaGene image analysis software (BioDiscovery, Los Angeles, CA, USA), and were analyzed with the GeneSight software package. For each spot, analyses were performed with mean values for signal intensity. For each slide, the local background was subtracted from the signal intensity, and the minimum intensity was raised to 20 by using a "floor" function. The ratio of mean hybridization intensities for each element was normalized by dividing by the mean of the selected subset (β-tubulin and actin-2). For duplicate spots on a single slide, the mean of two elements was used to combine them. To compare relative expression, data from multiple experiments were collected and hierarchical clustering was conducted. An average-linkage clustering algorithm was chosen for this analysis.

Results

cDNA Microarray Analysis and Normalization

Gene expression in leaves was monitored from 3-week-old *Arabidopsis* plants that were exposed to 0°C for various time periods. Each treatment was repeated several times and leaves were pooled from at least nine plants per treatment to minimize the variation.

For each time point, four independent hybridization experiments were conducted both to reduce the inherent variability of the microarray assay (Lee et al. 2000) and to improve the reliability of our results. The first sets of slides were probed with cDNAs labeled with Cy-3 and Cy-5 dUTP; for the second set, labeling was reversed for each sample pair to avoid potential artifacts caused by dye-related differences in labeling efficiency. The third and fourth sets of slides were hybridized using samples treated on different dates. The correlation coefficient among our four arrays was about 0.8 in every experiment, indicating that the reproducibility of arrays was within a reasonable range.

Normalization methods using all genes on an array generally assume that the typical RNA transcript is unresponsive to a given test condition relative to the control, and that a responsive gene is distinguishable from an unresponsive gene (Schena et al. 1995). These assumptions appear robust for genome-wide arrays, which comprise thousands of elements representing a large portion of the genome. However, the same assumption cannot be

applied to small-scale specialized arrays such as ours, where a large proportion of elements may reasonably be expected to change in response to the given stimulation. Therefore, in this study, instead of using global mean or median values, we included two housekeeping genes—β-tubulin and actin-2—for normalization. A normalization factor, calculated so that the mean ratio of those two genes was 1, was then applied to all the data. When such a normalization factor was used and analyzed for other housekeeping genes in this array, mean ratios of 0.83~1.22 were obtained at all time points (data not shown). This demonstrated that our normalization method was appropriate for further analysis.

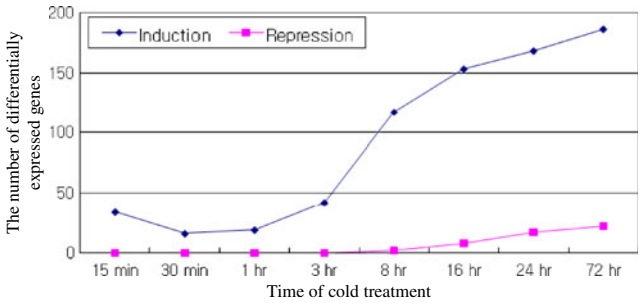

Fig. 1 Total number of differentially transcribed genes plotted at eight time points. Only those up- or down-regulated at least twofold were scored

Time Course of Gene Expression

Results from our microarray analysis also revealed many previously reported cold-regulated genes, thereby validating our microarray technique. Genes such as *cor*, *lea*, *rd*, and *CBF3* exhibited predicted patterns of expression (Table 1). That is, *cor* tended to be highly induced at the late phase while those reported to be induced early, such as the transcriptional activator *CBF3/DREB1a* (Thomashow 2001) were induced within 15 min of transfer to cold conditions.

Only genes that were up- or down-regulated by a factor of two or more were scored as significantly differentially regulated; most were differentially expressed at the later phase (Fig. 1). This result was not surprising because the probes included in our analysis were those previously detected by SAGE and SSH analyses of leaves and pollen cold-treated for 72 h. Distribution patterns at each time point were illustrated with a scatter plot (Fig. 2). Genes that showed differential expression soon after treatment were rarely induced or repressed by more than twofold whereas more genes were either induced or repressed to a greater extent as time passed.

Cluster Analysis of Microarray Data

To explore the relationship among samples and underlying features of gene expression, we compared their profiles via hierarchical clustering, a popular mathematical technique that attempts to group genes into small clusters and then assign those clusters to higher-level systems. This allows researchers to identify genes with related expression patterns and is also used to present profiles for individual genes during several different experiments.

We first drew separate histograms for each time point and then overlaid them (Fig. 3). Genes whose expression

Table 1 Microarray results of 11 well-known cold-induced genes

Clone	Fold change after cold treatment (0°C)								Accession	Locus name	Reference
	15 min	30 min	1 h	3 h	8 h	16 h	24 h	72 h			
LIU1 (cor15a)	1.81	1.75	1.59	4.90	24.17	64.66	114.98	133.04	AF325007	At2g42540	Thomashow 2001
LIU15 (cor15b)	3.44	3.13	2.87	2.51	10.33	10.83	10.99	8.37	L24070	At2g42530	Zhu 2001
LIU20 (L) (LEA D113 Type1)	1.59	1.49	1.48	1.09	11.26	48.27	48.68	38.50	X91915	At5g06760	Zhu 2001
LIU31 (WCOR413-like)	2.44	2.74	1.47	1.61	4.52	28.73	19.15	12.18	AF283004	At2g15970	Zhu 2001
G10 (chalcone synthase)	0.68	0.73	1.15	1.19	2.02	3.16	4.11	21.04	Z17649	At4g22880	Zhu 2001
LIU60 (Athb-12)	1.15	0.67	1.16	2.17	8.20	15.21	21.64	7.82	AF001949	At3g61890	Zhu 2001
LIU98 (cor47)	1.27	1.18	1.37	3.36	8.75	21.62	36.21	11.74	AB004872	At1g20440	Thomashow 2001
LIU132 (FL3-5A3)	1.18	1.26	0.97	1.30	3.60	11.21	14.10	8.87	AB044404	At2g15970	Seki et al. 2001
DREB1A (CBF3)	3.03	11.03	9.33	36.57	42.45	30.01	14.09	5.55	AB007787	At4g25480	Zhu 2001
LTI65 (RD29B)	2.32	2.72	1.29	1.70	3.50	7.26	14.71	9.69	X67670	AT5G52300	Zhu 2001
KIN 1	1.00	1.02	0.98	1.65	3.90	7.67	10.15	8.69	X51474	At5g15960	Zhu 2001

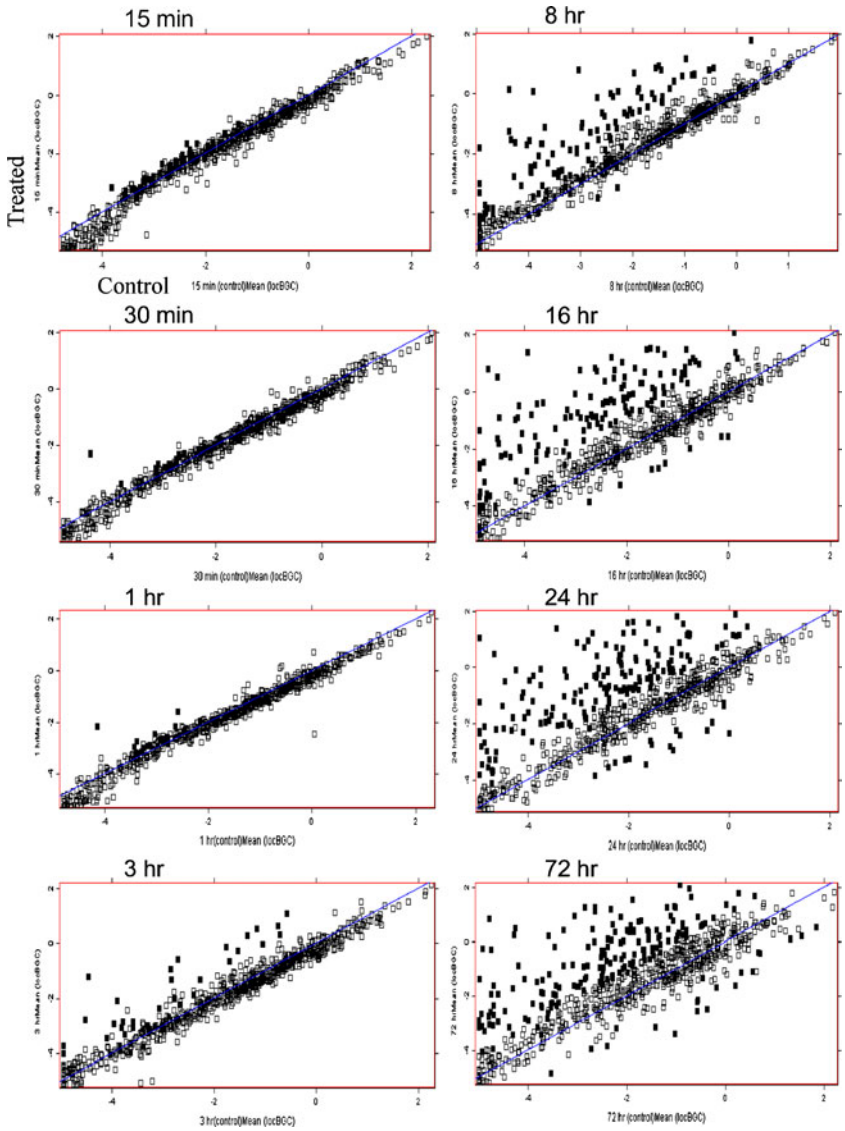

Fig. 2 Time course for gene expression in *Arabidopsis* leaves. Ratios are represented as scatter plots

differed by twofold or greater at any time point were selected from those plots for further analysis. Based on that criterion, 264 genes were induced and 33 were repressed. Those 297 genes were arranged into 10 groups according to their expression patterns.

Figure 4 shows the result of this hierarchical clustering analysis on the left side (red color for induction, green for repression). A few distinct patterns of expression were evident, as indicated by a simplified graphic form on the right. Those 10 groups could be characterized as follows: Type 1, genes induced within 15 min and rapidly repressed; Type 2, induced within 30 to 60 min before decreasing; Type 3, expression induced within 15 min and maintained for a while before declining; Type 4, induced highly at approximately hour 3, then continuing to be induced at the same or slightly elevated level throughout the late phase; Type 5, induced highly at approximately hour 8, then remaining constant or slightly increasing throughout the late phase; Type 6, induced in the late phase at a low level; Type 7, induced highly at approximately hour 16, then remaining constant or slightly increasing throughout the late phase; Type 8, induced at the end of the late phase at a low level; Type 9, having oscillating expression; and Type 10, having repressed expression in the late phase. In general, groups of differentially expressed genes tended to shift at 3∼8 h after cold treatment, making it reasonable to assume that expression occurred in two phases, i.e., early and late (before or after 3 h, respectively). Those induced early (Types 1, 2, and 3) were detected at relatively low levels, whereas the "late" genes comprised two groups, those highly induced (Types 4, 5, and 7) and those only slightly induced (Types 6 and 8).

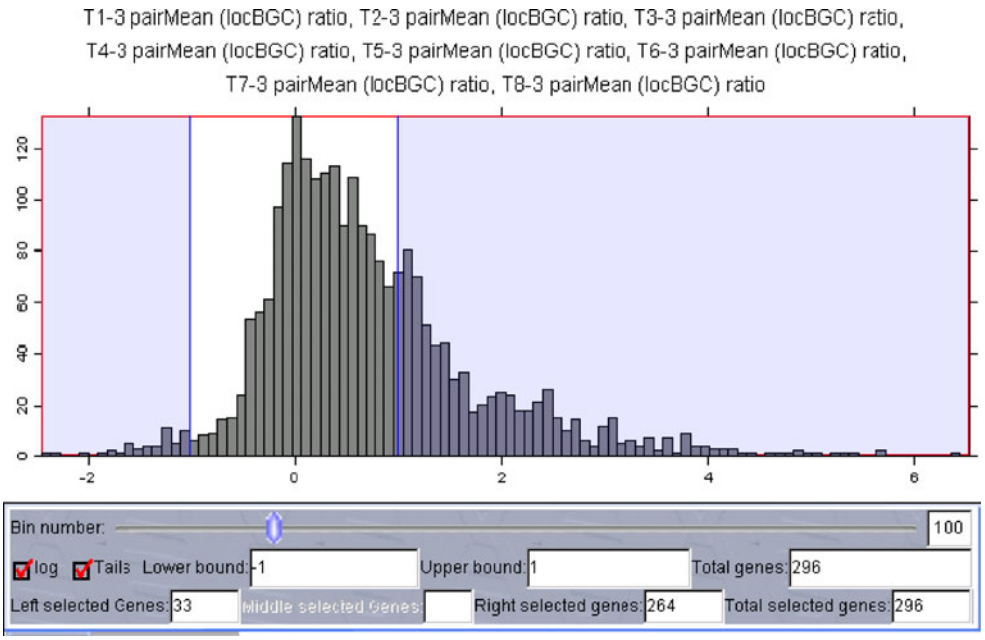

Fig. 3 Distribution of differentially expressed genes by cold treatment. Histograms for each time point were overlaid to select genes with twofold or greater differential expression under at least one experimental condition. Plot is log-transformed, with lower and upper bounds selected as −1 and 1, respectively

Approximately 40% of the genes within Types 4, 5, and 7 have previously been reported as cold-inducible, including *cor15a*, *cor15b*, *cor47*, *kin1*, *lti45*, and *lti40.* Therefore, the groupings that resulted from our current analysis demonstrate that our microarray data provide reliable evidence for the identification of COR genes because of the close agreement with that earlier research.

We also found several new genes, including *CBF3*, a major transcriptional activator that binds to the CRT/DRE. This gene, from Type 4, was induced here within 15 min of chilling exposure and was elevated for up to 8 h before slowly decreasing. Although *CBF2*, from Type 9, was induced in the same manner within 15 min, its expression level was less than for *CBF3*.

Genes that encode a lipid transfer protein (LIU19(L)) and fatty acid desaturase (LIU16) were also induced by cold stress. That response could be expected because lipid composition is necessarily altered in order for plants to acclimate to chilling. Here, LIU16, in Type 5, was highly induced beginning at hour 8 and expression peaked at hour 16. In contrast, expression of LIU19(L), from Type 7, remained constant for the first 16 h before rapidly increasing after 72 h.

We also identified a group of proteins involved in dehydration. S20 and LIU6, which encode dehydrin (Xero2/lti30), were rapidly induced at 3 h and transcripts continued to accumulate throughout the treatment period. Both belonged to Type 4. By contrast, dehydrin RAB18-like protein (LIU8) and LEA D113 Type 1 (LIU20(L)), from Type 7, were induced later, at approximately hour 16.

The contribution of some genes induced here by cold stress may not be considered direct. For example, genes with enzymatic functions, e.g., chalcone synthase (G10), alcohol dehydrogenase (L48-1), and pyrroline-5-carboxylate synthetase (LIU30-1/LIU30-2), were induced in the late phase, and were grouped within Type 7. However, results from previous genetics studies have suggested that these are not considered important for conferring freezing tolerance (Jarillo et al. 1993; Leyva et al. 1995).

Genes induced highly at approximately hour 8 (Type 5) included those that encode β-amylase (S48, S49, and S67). Glucose produced by β-amylase can be converted into sucrose and some other simple sugars. Although several roles for sugars in protecting cells from freezing injury have been proposed (Lineberger and Steponkus 1980; Steponkus 1984; Carpenter et al. 1986), soluble sugars alone are insufficient for providing complete freezing tolerance (Hincha et al. 1996).

In this analysis, 53 genes were newly described. Among them, 33 had already been identified but were not previously considered cold-inducible. The remaining 20, including LIU40-1 and LIU40-2, are novel genes not currently matched to database or putative protein sequences.

Finally, we found only a few genes (Type 10) that were down-regulated by cold stress, and most are related to photosynthesis. Their expression was repressed in the late phase.

Discussion

We used specialized microarrays comprising 712 cDNAs for time-course experiments to elucidate the pathways that mediate cold acclimation and for identifying new candi-

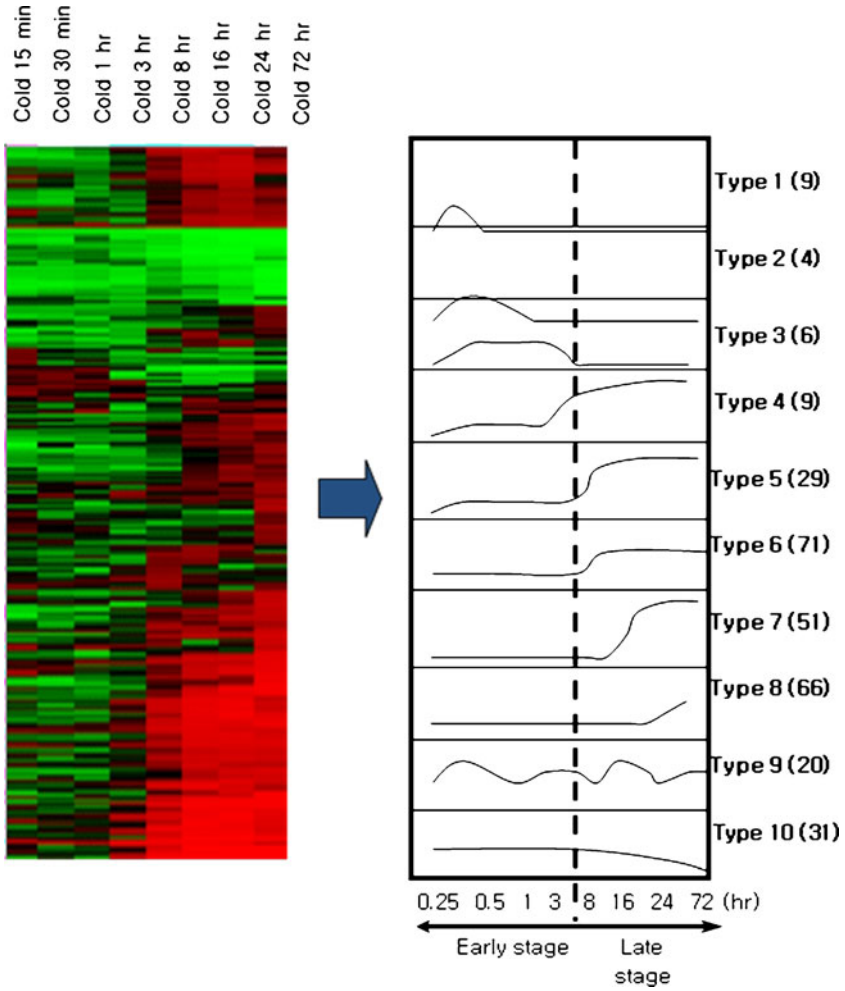

Fig. 4 Clustering analysis of time-course data for 264 selected genes with twofold or greater differential expression under at least one experimental condition. Hierarchical clustering was applied using average cluster linkage algorithm. Because patterns of differentially expressed genes tended to changed at 3~8 h post-treatment, line of demarcation was drawn between hours 3 (early) and 8 (late). Based on clustering results, 10 classification types were determined. Values in *parentheses* indicate numbers of genes per type

dates essential to freezing tolerance. In all, expression was altered (up or down) by at least twofold for 264 cold-inducible genes and 33 repressed genes. These genes were classified into 10 types according to the pattern of their expression in response to cold treatment. Those induced in the early phase (Types 1, 2, and 3) accounted for only 6% of the total. That outcome was lower than expected considering that the probes on the microarray originated from a list of COR genes. However, the probes spotted on the array were ones selected from a pool of genes we had previously found to be differentially regulated by 72 h of chilling. One of our study objectives was to determine how early those genes are induced. Here, many genes thought to be involved in cold acclimation were induced after approximately 8 h of exposure to cold temperature. We might reasonably presume that additional genes with the same expression pattern could be implicated in the same process. Furthermore, although many genes induced in the late phase might have a significant role in freezing tolerance, it is not yet possible to discern whether the early-responsive genes detected in our analysis are either expressed simply as a result of low-temperature stimulation or because they are truly related to cold acclimation.

The extent of induction or repression for an individual gene by specific stimuli depends on a complex interaction between its transcription apparatus and associated regulatory sequences. For example, a 9-bp conserved sequence, TACCGACAT, named the dehydration-responsive element (DRE) and DRE-related core motifs (CCGAC), is found in the promoter regions of many cold-inducible genes, e.g., *kin1*, *kin2*, *rd17/cor47*, and *cor15a* (Baker et al. 1994; Wang et al. 1995; Iwasaki et al. 1997). In addition, many cold-inducible genes that are also induced by exogenous ABA treatment contain potential ABA-responsive elements (ABREs) in their promoter regions (Ingram and Bartels 1996; Thomashow 1999). A cDNA microarray experiment with 1,300 full-length *Arabidopsis* cDNA clones (Seki et al. 2001) and a genomics study (Chen et al. 2002) that utilized the *Arabidopsis* GeneChip system with 402 genes that code for known and putative transcription factors have shown that the ABRE-like element and the DRE-like element

Table 2 ABRE, DRE, and CCGAC core sequences in the promoter regions of cold-inducible genes identified by cDNA microarrays

Gene[a] (locus name)	ABRE[b] (C/G/T)ACGTG(G/T)(A/C)	DRE[b] TACCGACAT	CCGAC core Motif[b] CCGAC	Identity	Status
LIU31 (At2g15970)	CACGTGGC (−74 to −67)[c]	TACCGACAT (−415 to −407)	TGCCGACAT (−806 to −798)	COR413. cold-acclimation protein, putative (FL3-5A3)	Reported
LIU1 (At2g42540)	CACGTGGC (−132 to −125)	–	GGCCGACCT (−184 to −176) GGCCGACAT (−361 to −353)	Cold-regulated protein cor15a precursor	Reported
LIU5 (At4g14690)	GACGTGGC (−494 to −487)	–	TACCGACAC (−454 to −446)	ELIP2. chlorophyll A–B binding family protein/early light-induced protein	Reported
LIU12 (At5g17460)	TACGTGGA (−82 to −75)	–	GGCCGACAT (−140 to −132)	At5g17460 (At5g17460/K3M16_30) mRNA	New
G76 (At3g59350)	TACGTGGA (−914 to −907)	–	AACCGACCA (−273 to −265)	Protein kinase-like protein Pto kinase interactor 1	New
LIU15 (At2g42530)	CACGTGTA (−71 to −64)	–	GGCCGACCT (−195 to −187)	Cold-regulated protein cor 15b precursor	Reported
LIU119 (At5g01600)	CACGTGGA (−74 to −67) TACGTGGA (−664 to −657) GACGTGTA (−731 to −724)	–	CTCCGACGT (−335 to −347)	Ferritin 1 precursor	Reported
L102-1 (At1g69870)	TACGTGGA (−201 to −194)	–	GACCGACAA (−343 to −335)	Proton-dependent oligopeptide transport (POT) family protein	New
G54 (At1g61800)	TACGTGTA (−594 to −587)	–	–	Glucose-6-phosphate/phosphate-translocator precursor	New
G139 (At5g42900)	CACGTGTA (−66 to −59) GACGTGGC (−83 to −76)	–	–	Putative protein similar to unknown protein (pir||T05226)	New
G26 (At1g06040)	TACGTGTA (−479 to −472)	–	–	Salt-tolerance protein	New
LIU118 (At4g27410)	GACGTGGC (−157 to −150) CACGTGTC (−481 to −474) CACGTGTA (−763 to −756) TACGTGTC (−920 to −913)	–	–	RD26. no apical meristem (NAM) f amily protein (RD26)	New
L48-1 (At1g77120)	CACGTGGA (−219 to −212)	–	–	Alcohol dehydrogenase	Reported
LIU52 (At3g62980)	CACGTGTC (−80 to −73)	–	–	Transport inhibitor response 1 (TIR1)	New
LIU78 (At5g05270)	CACGTGGC (−101 to −94)	–	–	Chalcone-flavanone isomerase family protein	New
G32 (At5g25110)	TACGTGTA (−866 to −859)	–	–	CBL-interacting protein kinase 25 (CIPK25)	New
G14 (At2g45040)	CACGTGTC (−231 to −224)	–	–	Matrix metalloproteinase	New
LIU6 (At3g50970)	–	–	CACCGACGT (−238 to −230)	Dehydrin (Xero 2)	Reported
LIU92-3 (At5g61790)	–	–	GTCCGACTT (−935 to −927) GCCCGACAT (−143 to −135)	Calnexin 1 (CNX1)	New
G58 (At4g39730)	–	–	TACCGACTC (−205 to −197)	Lipid-associated family protein	New
LIU60 (At3g61890)	–	–	AACCGACCT (−861 to −853)	Homeobox-leucine zipper protein ATHB-12	Reported
LIU54 (At4g27520)	–	–	–	ENL2. Plastocyanin-like domain-containing protein	New
LIU68 (At2g02100)	–	–	–	Protease inhibitor II	New

Table 2 (continued)

Gene[a] (locus name)	ABRE[b] (C/G/T)ACGTG(G/T)(A/C)	DRE[b] TACCGACAT	CCGAC core Motif[b] CCGAC	Identity	Status
G69 (At2g16890)	–	–	–	UDP-glucoronosyl/UDP-glucosyl transferase family protein	New
G75 (At1g73390)	–	–	–	Unknown protein (At1g73390) mRNA	New
LIU56/S75 (At2g28900)	–	–	–	ATOEP16. mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family	New
G117 (At4g35590)	–	–	–	RWP-RK domain-containing protein	New
LIU21 (At2g46680)	–	–	–	Homeodomain transcription factor (ATHB-7)	New
LIU25 (At5g13170)	–	–	–	Nodulin MtN3 family protein	New
LIU40-1/LIU40-2 (At4g38580)	–	–	–	Farnesylated protein (ATFP6)	New

[a] Genes represent those of Types 4, 5, and 7 in Fig. 4, whose expression ratios are increased more than twofold by cold-stress treatments

[b] ABRE, DRE, and CCGAC core sequences observed in 1,000-bp upstream regions of the 5′ termini from isolated cDNA clones.

[c] Numbers in parentheses indicate the nucleotide beginning at the 5′ terminus of the isolated cDNA clone. Minus signs indicate that the nucleotide exists upstream of the 5′ terminus for the putative transcription start site.

occur at significantly higher frequencies in the promoters from cold-responsive genes.

When we examined the promoter sequences of 30 genes from Types 4, 5, and 7, we found that 21 contained those elements (Table 2). To analyze the promoter region of target genes, we used sequences located 1.0 kb upstream from the 5' termini of each RIKEN *Arabidopsis* full-length (RAFL) cDNA clone (Seki et al. 2002b). Those cold-inducible genes were organized into four groups according to the components of their promoter regions. The first comprised eight genes with both ABRE and DRE-like elements; three of those genes were newly determined. It is possible that these Group-1 genes might be controlled by both ABA-dependent and ABA-independent pathways. Genes in the second and third groups (13 total, 10 new) had either an ABRE or a DRE-like element. Group 4, with nine genes, had neither element. Many CRT/DRE-controlled genes are induced by the CBF-mediated ABA-independent pathway; numerous COR genes, by the bZIP-mediated ABA-dependent pathway (Gilmour and Thomashow 1991; Nordin et al. 1991; Mantyla et al. 1995). However, it is believed that at least two other signal-transduction pathways exist for cold acclimation (Xin and Browse 2000). The fact that we found some genes whose promoter had neither ABRE nor DRE-like elements suggests that novel *cis*-acting elements might occur in cold-inducible gene promoters.

Here, we detected several cold-inducible genes also reportedly related to other stress responses, such as salt-tolerance protein G26, the water stress-induced protein-like protein mRNA for LIU83-1/LIU83-2, and a stress-related protein gene for L103-1. This observation supports the hypothesis that networks of multiple signaling pathways mediate plant responses to salt, water, or cold stress (Zhu 2001). Although those kinds of stresses clearly differ in their physical natures, and each elicits specific plant responses, they also activate some common reactions in plants whose genes are induced by all three kinds (Shinozaki and Yamaguchi-Shinozaki 1997). We also identified two defense-related genes—thaumatin-like protein (LIU45) and putative glutathione S-transferase (LIU58). The former shows high similarity to the pathogenesis-related (PR)-5 protein and the latter is supposedly involved in the detoxification of potentially genotoxic chemicals. Finally, our finding of LIU25 and L109-1, both senescence-related protein genes, supports the hypothesis that the signal pathway activated by senescence may overlap substantially with stress-signaling pathways (Chen et al. 2002).

In conclusion, we have used a combination of SAGE and microarray analyses to find a large number of genes not previously described as cold-regulated. Cloning and characterization of these new genes may enhance our understanding

of their cold-stress regulation as well as provide a tool for their application in plant research. Our results also demonstrate that such an analytical technique can be more useful than conventional methods, e.g., differential screening, for the selection of new candidate genes responsible for cold acclimation.

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