RNA Expression Profiles and Data Mining of Sugarcane Response to Low Temperature[1]

Fábio T.S. Nogueira, Vicente E. De Rosa Jr., Marcelo Menossi, Eugênio C. Ulian, and Paulo Arruda*

Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, 13083–970, Campinas, SP, Brazil (F.T.S.N., V.E.D.R., M.M., P.A.); Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas, 13083–970, Campinas, SP, Brazil (F.T.S.N., V.E.D.R., M.M., P.A.); and Centro de Tecnologia Copersucar, 13400–970, Piracicaba, SP, Brazil (E.C.U.)

Tropical and subtropical plants are generally sensitive to cold and can show appreciable variation in their response to cold stress when exposed to low positive temperatures. Using nylon filter arrays, we analyzed the expression profile of 1,536 expressed sequence tags (ESTs) of sugarcane (*Saccharum* sp. cv SP80-3280) exposed to cold for 3 to 48 h. Thirty-four cold-inducible ESTs were identified, of which 20 were novel cold-responsive genes that had not previously been reported as being cold inducible, including *cellulose synthase*, ABI3-interacting protein 2, a negative transcription regulator, *phosphate transporter*, and others, as well as several unknown genes. In addition, 25 ESTs were identified as being down-regulated during cold exposure. Using a database of cold-regulated proteins reported for other plants, we searched for homologs in the sugarcane EST project database (SUCEST), which contains 263,000 ESTs. Thirty-three homologous putative cold-regulated proteins were identified in the SUCEST database. On the basis of the expression profiles of the cold-inducible genes and the data-mining results, we propose a molecular model for the sugarcane response to low temperature.

Cold is one of the most important environmental stresses affecting plant growth and crop productivity. Chilling (low temperatures above 0°C) and freezing (temperatures below 0°C inducing extracellular ice formation) limit the geographical distribution and growing season of many crops and cause significant crop losses (Xin and Browse, 2000). However, chilling and freezing stresses differ from each other in that the former involves a direct effect of low temperature on cells, whereas freezing often acts indirectly, damaging cells by dehydration (Pearce, 1999).

Plants vary considerably in their ability to survive under chilling and freezing temperatures. At one extreme, some herbaceous plants from temperate regions can survive under freezing temperatures ranging from −5°C to −30°C. At the other extreme, plants from tropical and subtropical regions have virtually little or no capacity to survive even the slightest freezing (Thomashow, 2001). In addition, even species considered to be sensitive to chilling can show substantial variation in their response to colder temperatures. Cold (chilling and freezing) tolerance often increases if plants are first hardened by exposure to a period of acclimation at low positive temperatures (Thomashow, 2001). Cold acclimation is associated with biochemical and physiological changes that include alterations in carbohydrate metabolism, membrane lipid composition, phenylpropanoid content, respiration, photosynthesis, and oxidative stress defenses (Allen and Ort, 2001). In general, plants exposed to low temperatures show two basic responses: the adjustment of metabolism to kinetic constraints imposed by low temperatures, and the induction of tolerance to chilling and freezing via the activation of specific genes. These alterations are frequently caused by changes in gene expression (Guy et al., 1985), as shown by the isolation and characterization of several cold-regulated (*COR*) genes from different plant species (Thomashow, 1998).

Cold-induced genes can also be induced by water stress and, in several cases drought- and cold-inducible genes are also induced by the phytohormone abscisic acid (ABA). Dehydration caused by water stress or cold appears to trigger ABA biosynthesis, which in turn induces the expression of several genes (Liu et al., 1998). However, an ABA-independent pathway of gene induction by low temperature and water stress has been described (Yamaguchi-Shinozaki and Shinozaki, 1994; Gilmour et al., 1998; Liu et al., 1998). Direct evidence of an ABA-independent pathway came from the identification of three cold-induced transcription factors (*CBF1*, *CBF2*, and *CBF3*) from Arabidopsis that encode proteins containing AP2 DNA-binding motifs (Medina et al., 1999). These transcription factors are not induced by exogenous ABA, suggesting that they participate in a cold-induced ABA-independent

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* Corresponding author; e-mail parruda@unicamp.br; fax 55–19–3788–1089.

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pathway (Yamaguchi-Shinozaki and Shinozaki, 1994; Seki et al., 2001). These factors bind to the dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element present in the promoter of drought-, high salt-, and cold-inducible genes (Liu et al., 1998). Constitutive overexpression of *CBF1* and *CBF3* genes in nonacclimated transgenic Arabidopsis plants induces the expression of several cold-inducible genes, thereby increasing the tolerance of the plant to freezing (Gilmour et al., 2000).

Sugarcane (*Saccharum* sp.) is generally considered as a cold-sensitive plant (Tai and Lentini, 1998). The magnitude of cold (chilling and freezing) damage is dependent on the severity and duration of the low temperature, cultivar resistance to post-freezing deterioration, and time lapse and temperature fluctuations between the freeze event and harvest (Tai and Lentini, 1998). However, field observations have shown that the sensitivity of sugarcane to cold varies among varieties. Du et al. (1999) demonstrated that some subtropical hybrid species are more cold tolerant than tropical species. Thus, the identification of sugarcane cold-responsive genes could be an important resource in breeding programs to detect genetic differences among hybrids and to obtain transgenic plants with improved tolerance to cold and its related stress.

The rapid advance of genome-scale sequencing has led to the development of methods for analyzing transcript abundance in a large set of genes involved in abiotic stress responses (Perret et al., 1998; Schena et al., 1998; Schummer et al., 1999; Freeman et al., 2000; Seki et al., 2001; Fowler and Thomashow, 2002). Expressed sequence tags (ESTs) are a rich source for gene discovery in several organisms. EST databases can be used for large-scale data mining of genes involved in specific pathways and, in association with techniques for expression profile analysis, can be helpful in analyzing the global response of tissues or whole organisms under biotic or abiotic stress and in the discovery of novel genes (Cushman and Bohnert, 2000). In this work, we describe the use of sugarcane ESTs from the Sugarcane EST Genome Project (SUCEST; http://sucest.lad.ic.unicamp.br) to construct high-density filter arrays containing random ESTs to identify cold-responsive genes. Using the expression profile data generated and extensive data mining in the SUCEST database, we constructed a putative model for global sugarcane gene expression under cold exposure.

RESULTS

Construction of Sugarcane EST Macroarrays and Data Analysis

Using SUCEST cDNA clones and a hand-held tool with a 96-pin printhead (V&P Scientific, San Diego, CA), we constructed sets of two high-density filters, each filter containing 768 random EST targets, thereby totaling 1,536 ESTs. The macroarray nomenclature used was that established for filter-based methods in which the target is the DNA spotted onto the filter surface, and the probe is the labeled DNA that is hybridized to the surface-bound DNA (Rose, 2000).

To decrease variation in the amount of DNA among spots and filters, each EST clone was spotted twice at the same position on all filters. This procedure reduced the coefficient of variation (cv) among spots by 50% (J.M. Felix, R. Drummond, F.T.S. Nogueira, R.A. Jorge, P. Arruda, and M. Menossi, unpublished data). Each EST was spotted at two positions on the filters to assess the reproducibility of spotting.

Before cDNA probe hybridization, the high-density filters were hybridized with a probe corresponding to the plasmid vector (see "Materials and Methods"), and the signal intensity was measured. The median value for all spot intensities of each filter was determined and then we estimated the cv of these median values to assess fluctuation in the amount of DNA among replicate filters. Only replicate filters with cv values lower than 10% were used for subsequent analysis. In addition, to reduce the variation among replicate filters caused by differences in the experimental conditions, the average of all signal intensities obtained with the cDNA probe from each filter was set to 1 (Schummer et al., 1999). To assess the signal variation between replicate spots, we estimated the ratio between the signal intensities of the two replicate spots representing each EST spotted onto filters. Around 98% of ESTs had ratios ranging from 0.5 to 2.0, indicating that the DNA variation between replicate spots was less than 2-fold for most ESTs (data not shown). This finding agreed with data for nylon filter arrays spotted with a manual device (Schummer et al., 1997). Finally, 12 spots representing the empty plasmid vector were used in each filter as a negative control to assess nonspecific hybridization. These spots consistently produced a hybridization signal above background, probably because of some degree of similarity between sugarcane cDNA and the vector sequences. Thus, only those sugarcane ESTs displaying signal intensities higher than the average signal intensity of the negative control plus two SD for three or more arrays were considered for further data analysis.

Identification and Functional Classification of Cold-Responsive Sugarcane ESTs

To determine the threshold for changes in gene expression that could be attributable to cold treatment, we used the strategy reported by Friddle et al. (2000) in which the $\log_2$ of the expression ratios (i.e. ratios between the normalized signal intensity of each time point for treated and control samples of each EST) were normally distributed and centered on

a ratio of 0 (i.e. the average of the expression ratios was 1). The average and the SD of the expression ratios ($\log_2$ transformed) for each cold-treatment time point were estimated from two independent experiments. Comparison of the ratio of signal intensities between two independent, untreated control probes yielded an *R* value of around 0.92, indicating a good correlation between replicate experiments. Assuming that experimental variation was nonspecific for any particular EST, we selected as the threshold of significance an expression ratio at least 1.65 SD above or below the average expression ratio for each cold-treatment time point in two replicate experiments ($P < 0.0025$). Moreover, among the previously selected ESTs, we only considered those displaying at least a 2-fold induction or repression at each interval of cold treatment relative to the control. A representative filter comparing control and cold-treated plantlets samples (48-h time point) is shown in Figure 1. Fifty-nine high quality sugarcane ESTs in the SUCEST database showing significantly altered expression during cold treatment were identified, of which 34 were up-regulated by cold treatment. Scatter plots of the expression ratios of cold-treated versus control plantlets are shown in Figure 2. Ratios below 1.0 were inverted and multiplied by −1 to aid data interpretation (Girke et al., 2000). In most ESTs, expression was unchanged by cold treatment (Fig. 2). The expression of a few ESTs was altered after 6 h of cold treatment, whereas the highest induction or repression occurred after 24 h of cold exposure. Figure 3 shows the expression profiling of a randomly sampled subset of six cold-inducible sugarcane ESTs from two independent experiments. Although the absolute -fold induction values were not identical between biological samples, the expression profiles were similar, corroborating the reproducibility of our array data.

The usefulness of our arrays for screening *COR* genes was demonstrated by the identification of several cold-inducible genes that had already been reported for other plants (Tables I and II). The putative relevant biological functions of all cold-responsive sugarcane ESTs identified are shown in Table II. The sugarcane cold-inducible ESTs were distributed in three classes (Table I). The first class contained 14 ESTs with homologs in other organisms in which they represent drought and cold-inducible genes. These included xanthine dehydrogenase (*XDH*), ocs-element binding factor 1 (*OCSBF-1*), pyruvate orthophosphate dikinase (*PPDK*), superoxide dismutase (*SOD*), NADP-dependent malic enzyme (*NADP-ME*), a putative sugar transporter, *polyubiquitin*, and NAC genes (Table II). The second class consisted of seven ESTs not previously described as being induced by cold stress. Among them were an EST encoding ABI3-interacting protein 2 (AIP2) involved in development (Kurup et al., 2000) and an EST encoding cellulose synthase (Table II). The third class contained 13 ESTs encoding unknown proteins with no hits in the GenBank nr database (Table II).

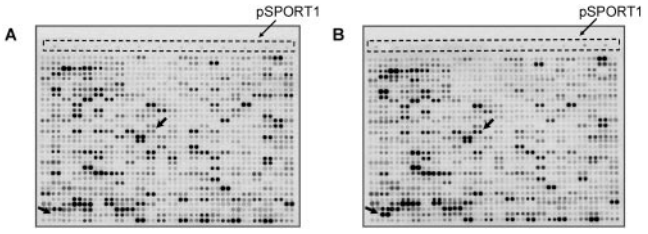

Figure 1. Examples of macroarray filters used to analyze gene expression under cold acclimation. Filters containing 768 random sugarcane cDNAs in duplicate were probed with [^{33}P]cDNA reverse-transcribed from total RNA of control (A) and cold-treated (48 h at 4°C; B) sugarcane plantlets. Nor-specific hybridization was monitored using 12 spots representing the empty pSPORT1 vector (dashed rectangles). The signals were detected in a phosphorimager analyzer. The arrows indicate examples of cold-inducible sugarcane ESTs.

Among the total cold-responsive ESTs, 25 (47%) were down-regulated by cold exposure, 13 of which encode proteins presenting no hit in the GenBank nr database (Table II). Twelve ESTs encode proteins with a wide range of functions, including transcription, signaling (receptor-like protein kinases), amino acid metabolism (acetohydroxyacid synthase and Asn synthetase), defense (pathogenesis-related protein) development (NAM-like protein), and water status (aquaporin). These results suggest that several metabolic processes, including perception of stress signals and regulation of gene expression, were repressed during cold stress.

To estimate the relative contribution of cold-inducible genes from each SUCEST library used in the array experiments, we first calculated the normalized number of cold-inducible ESTs (i.e. the ratio between the number of cold-inducible ESTs from each library and the total ESTs spotted onto filters for each SUCEST library) and then estimated the percentage of cold-inducible ESTs from each cDNA library (Fig. 4). Interestingly, the library HR1 (Vettore et al., 2001) accounted for most of the cold-inducible ESTs. This library was constructed from bacterium-infected sugarcane tissues (Vettore et al., 2001) and could be a good source for the discovery of biotic and abiotic stress-related genes.

RNA-Blot Analysis

To validate the macroarray data, we did blot analysis using total RNA from a new set of cold-treated and untreated plantlets. Five cDNA clones representing *polyubiquitin*, *OsNAC6*, and three novel cold-inducible genes were analyzed. Figure 5 compares the gene expression profiling obtained using macroarrays and RNA gel blots. Although the absolute -fold induction values of the blots were not identical to those on the array, there was a high consistency between the two data sets.

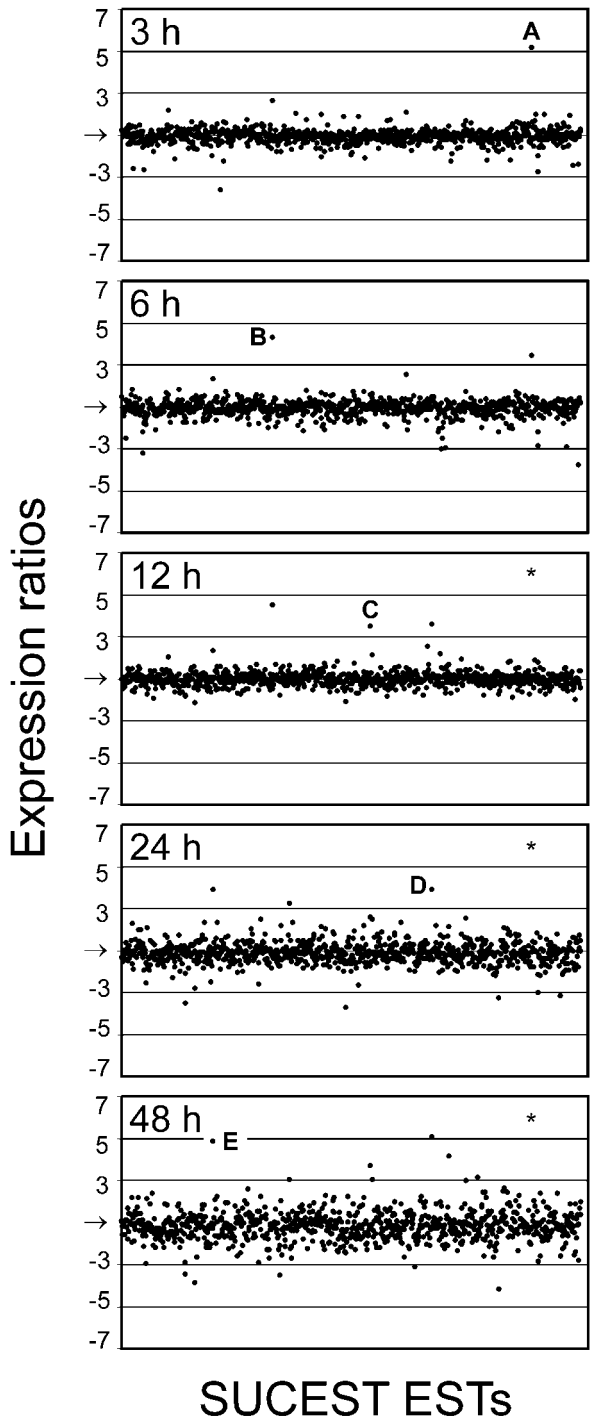

Figure 2. Scatter plots of the expression ratios of all ESTs analyzed. The average of the normalized relative intensities of each EST was used to calculate the expression ratios. The ratios that were higher with cold treatment (3, 6, 12, 24, and 48 h at 4°C) are plotted upward, and those that were higher in the controls are plotted downward. The lateral arrows represent ratios of approximately 1. Letters on the scatter plots indicate examples of cold-inducible sugarcane ESTs. A, EST encoding to no hit protein; B, *OsNAC6* gene; C, EST encoding to unknown protein; D, *OsNAC4* gene and E, *cellulose synthase* gene. Asterisks in the 12, 24, and 48 h panels indicate expression ratios that surpassed the ratio scale. Only one replicate macroarray experiment is represented in this figure.

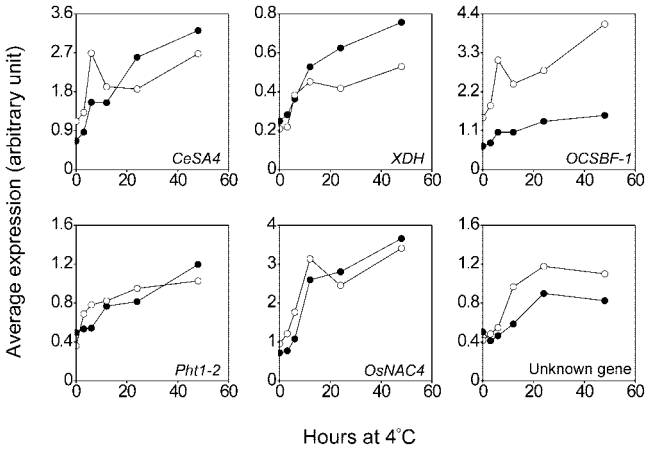

Figure 3. Examples of the expression profile of cold-inducible sugarcane ESTs. The average expression was calculated from normalized relative intensities of each time point of each EST from two independent experiments (white and black circles). *CeSA4*, Cellulose synthase 4; *Pht1-2*, phosphate transporter; and *OsNAC4*, rice NAC gene 4.

Data Mining and Domain Analysis

To complement the expression profiling data of cold-inducible genes obtained with macroarrays and to provide a global view of the up-regulation of gene expression in sugarcane during cold exposure, we undertook extensive data mining in the SUCEST databank to find homologs of cold-inducible genes reported in other plant species. Initially, we created a protein sequence database containing 250 cold-inducible genes reported in the literature and present in the GenBank database (http://www.ncbi.nlm.nih.gov). With the tBLASTN algorithm, these protein sequences were used as drivers to identify putative assembled sequences (combined set of contigs and singlets representing different transcripts from the SUCEST database). The criteria used to select the SUCEST-assembled sequences were the E value and percentage of protein coverage. Assembled sequences with an E value $\leq 10^{-20}$ and a protein sequence coverage greater than 70% were considered to represent putative cold-inducible gene homologs. Thirty-three SUCEST-assembled sequences encoding proteins similar to COR proteins described for other plants were identified (Table III). Interestingly, one

Table I. *Cold-inducible sugarcane ESTs identified by macroarray expression profiling*

Summary	No. of ESTs	% ESTs
Total cold-inducible ESTs	34	100
Cold- and drought-inducible ESTs[a]	14	41
ESTs unrelated to cold[b]	7	21
Novel cold-inducible ESTs[c]	13	38

[a]ESTs homologous to previously described cold- and drought-inducible genes. [b]ESTs homologous to previously described genes, but not induced by cold. [c]ESTs encoding proteins that have not been described previously.

Table II. *Average expression ratios and sequence similarities of cold-responsive sugarcane ESTs*

Clone Identification	BLAST Hit[a]	E Value	Description[b]	Ratios[c]				
				3 h	6 h	12 h	24 h	48 h
Up-regulated								
			Proteolysis					
SCEPCL6029G10	AAC16012	7E − 86	Polyubiquitin protein	1.1	1.4	2.1	2.0	3.4
SCCCHR1002E03	S28426	1E − 133	Polyubiquitin protein	1.0	1.2	1.8	2.5	3.0
SCCCAD1002H02	CAA66667	1E − 131	Polyubiquitin protein	0.8	1.1	1.7	2.0	3.0
SCCCHR1002A02	CAA66667	1E − 119	Polyubiquitin protein	0.9	1.2	1.6	1.6	2.8
SCEPCL6028G03	S17435	0.0	Polyubiquitin protein	1.0	1.2	1.6	1.7	3.0
			Plant development					
SCMCCL6027D02	BAA89800	1E − 141	OsNAC6 protein	2.2	3.3	4.0	6.2	8.5
SCCCHR1004F11	BAA89798	1E − 52	OsNAC4 protein	1.2	1.7	3.4	3.2	4.3
SCRLCL6032B05	BAB09485	3E − 16	NAM protein	1.0	1.0	2.0	2.1	2.6
			Antioxidant metabolism					
SCCCHR1004E11	T10235	0.0	Xanthine dehydrogenase	1.1	1.6	2.1	2.2	2.8
SCCCLR1068H03	P93407	1E − 42	Cu/ZnSOD protein	2.0	1.5	1.1	2.2	2.0
			Transcription regulation					
SCEPCL6021E11	P24068	3E − 57	OCSBF-1 protein	1.2	1.8	1.6	2.0	2.6
SCEPCL6028F01	BAA97498	2E − 21	Negative transc regulator	1.2	1.2	1.1	1.5	2.2
SCCCHR1003E12	CAB75509	2E − 26	AIP2 protein	2.1	1.4	1.1	1.0	0.9
			CO_2 fixation					
SCEPCL6023E08	CAA06247	0.0	PPDK protein	1.4	1.8	1.8	2.1	1.3
SCEPCL6023H04	AAK91502	0.0	NADP-ME	1.6	2.2	2.1	1.2	1.1
			Cell wall metabolism					
SCMCCL6027C02	AAF89964	0.0	Cellulose synthase-4	1.3	2.4	2.0	2.8	3.6
SCCCAD1003B03	T04331	2E − 75	se-wap41 protein	1.3	1.8	2.1	1.9	3.2
			Protein metabolism					
SCCCHR1003G02	AJ309824	0.0	25S ribosomal gene	1.3	1.2	1.4	2.8	3.2
SCCCAD1001H05	AJ309824	0.0	25S ribosomal gene	1.5	1.7	2.3	3.5	4.0
			Transporter					
SCCCAD1001G06	AAK25880	5E − 47	Putative sugar transporter	1.1	1.2	2.0	1.9	2.5
SCCCHR1003H07	AAM14593	0.0	Pht1-2	1.5	1.6	1.9	2.1	2.6
			Unknown/unclassified					
SCEPCL6021A10	T04466	1E − 94	Unknown protein	1.2	1.9	1.8	2.1	3.1
SCMCCL6027G02	AAD20139	3E − 54	Unknown protein	1.2	1.3	1.5	1.8	3.4
SCCCHR1003D12	T45625	5E − 30	Unknown protein	2.1	1.2	1.0	1.0	0.9
SCCCHR1004E10	AAD32824	3E − 55	Unknown protein	1.0	1.6	2.9	2.4	3.9
SCEPCL6028F02	AAD20391	2E − 45	Unknown protein	1.1	1.2	1.1	1.6	2.4
SCCCAD1003C07	BAB56055	4E − 19	Unknown protein	0.8	1.4	1.7	2.2	2.5
SCCCHR1003A12	NP_567065	3E − 13	Unknown protein	1.4	2.1	2.6	2.8	2.7
SCCCAD1002F02	NP_564419	3E − 12	Unknown protein	1.0	1.1	1.7	2.3	2.1
SCCCLR1068G12	BAB07950	4E − 24	Unknown protein	1.3	1.2	2.1	1.8	1.7
SCCCHR1003H01	–	–	No protein match	4.0	4.1	8.7	15.3	29.9
SCEPCL6028G02	–	–	No protein match	0.8	1.1	1.3	1.9	2.6
SCEPCL6029H09	–	–	No protein match	0.8	1.2	2.1	1.4	2.4
SCUTCL6036C05	–	–	No protein match	1.5	0.9	1.3	0.9	3.1
Down-regulated								
			Plant development					
SCRLCL6032B11	AAN64996	1E − 24	Putative NAM protein	0.7	0.4	0.7	0.8	0.6
			Transcription regulation					
SCRLCL6032F11	BAC55608	0.0	Scarecrow-like protein	0.7	0.5	0.7	0.6	0.5
			Signal transduction					
SCEPCL6028A06	AAK43512	1E − 141	Putative receptor kinase	1.2	0.4	1.1	0.9	1.1
SCRLCL6032H12	AAL67082	0.0	Putative receptor kinase	0.7	0.5	0.7	0.7	0.6

(*Table continues*)

CBF transcription factor was found among these 33 SUCEST putative genes (Table III). Recently, Jaglo et al. (2001) identified components of the CBF regulon (DRE/CRT-containing genes that are induced by CBF transcription factors) in other plant species. Thus, sugarcane may have novel genes in the CBF regulon with roles in mediating the responses to cold. In Arabidopsis, proteins encoded by the CBF regulon protect cells against freezing and other stress associated with dehydration (Thomashow, 2001).

Table II. *Continues*

Clone Identification	BLAST Hit[a]	E Value	Description[b]	Ratios[c]				
				3 h	6 h	12 h	24 h	48 h
			Amino acid metabolism					
SCCCHR1001G10	CAA45117	0.0	Acetohydroxyacid synthase	0.9	0.8	1.0	0.5	0.4
SCCCLR1068G05	AAK49456	1E − 159	Asn synthetase	0.7	0.5	0.9	0.8	0.6
			Water channel					
SCCCHR1004G12	AAK26758	1E − 163	Aquaporin	0.7	0.6	0.6	0.3	0.2
			Defense					
SCRLCL6032B09	T05694	6E − 67	Pathogenesis-related protein	1.2	0.4	1.0	1.4	1.0
			Heat shock response					
SCEPCL6023F09	CAA77978	0.0	HSP82	0.9	0.8	0.6	0.6	0.3
SCAGCL6016D10	CAA77978	0.0	HSP82	0.8	0.7	0.6	0.6	0.3
			Lipid metabolism					
SCRLCL6032B08	NP_178516	0.0	Acyl-CoA synthetase	0.7	0.5	1.0	0.9	0.6
			Protein metabolism					
SCCCHR1001C11	P49210	1E − 95	Ribosomal protein L9	0.9	0.7	0.7	0.4	0.4
			Unknown/unclassified					
SCEPCL6023A10	BAA92204	4E − 52	Unknown protein	0.9	0.9	0.5	0.5	0.5
SCEPCL6029A12	AAL36075	1E − 155	Unknown protein	0.4	0.3	1.2	0.5	0.4
SCCCHR1001B11	BAB64285	5E − 13	Unknown protein	0.7	0.5	0.4	0.2	0.2
SCCCHR1004B05	BAB64285	5E − 13	Unknown protein	0.8	0.5	0.4	0.3	0.2
SCCCAD1002A11	–	–	No protein match	0.6	0.6	0.4	0.2	0.3
SCEPCL6023A04	–	–	No protein match	1.3	0.3	1.4	1.3	1.2
SCAGCL6016A10	–	–	No protein match	0.4	0.9	0.9	0.7	0.6
SCUTCL6036B02	–	–	No protein match	0.9	1.0	0.5	0.5	0.4
SCCCHR1001B05	–	–	No protein match	0.6	0.4	0.4	0.2	0.2
SCRLCL6032D11	–	–	No protein match	0.6	0.4	0.7	0.6	0.4
SCRLCL6032E11	–	–	No protein match	0.6	0.4	0.7	0.8	0.5
SCRLCL6032G07	–	–	No protein match	0.8	0.4	0.8	0.9	0.6
SCRLCL6032E10	–	–	No protein match	0.7	0.5	0.8	0.7	0.5

[a]BLAST hit of each sequence was obtained using the BLASTX algorithm, except for the ribosomal genes, which were also analyzed by the BLASTN algorithm. [b]Description indicates the putative functions of the gene products expected from similarity sequences. [c]Each value represents the average of the expression ratios between the normalized relative intensity of each interval of cold treatment and the control (0-h exposure to cold) from two independent experiments.

To identify putative conserved domains, the inserts of all cDNAs encoding for unknown/unclassified cold-inducible proteins were completely sequenced. Most of the domains identified were related to proteins involved in the regulation of gene expression and signal transduction (Table IV). It is possible that these unknown proteins belong to novel cold-response pathways and cold/drought-tolerance. Finally, Figure 6 shows a possible panel of the up-regulation of gene expression during sugarcane cold adaptation, based on the cold-inducible gene expression profiling data and the data mining results described above.

DISCUSSION

Cold-Responsive Genes in Sugarcane

We employed high-density filters to assess the expression profile of sugarcane ESTs when plants were submitted to cold treatment for up to 48 h. Eleven of the cold-inducible ESTs found in our experiments represented genes reported to be induced by cold and drought in other plants. Acclimation to cold induces several biochemical and physiological alterations in the cellular machinery and probably improves plant tolerance to cold and other cold-related stress (Guy et al., 1985; Allen and Ort, 2001). Cold- and drought-related stresses induce a set of common genes (Liu et al., 1998). In this study, we identified five cold-inducible sugarcane ESTs encoding for polyubiquitin proteins (Table II). These proteins occur in all eukaryotes either as a free monomer or covalently linked to a variety of other proteins. One of their major functions is in tagging proteins for selective degradation by the 26S proteasome (O'Mahony and Oliver, 1999). As far as we know, there are no reports showing the induction of *polyubiquitin* gene expression by chilling or freezing in plants. However, O'Mahony and Oliver (1999) isolated a polyubiquitin cDNA involved in vegetative desiccation. Accordingly, it is possible that the late cold-induced expression of sugarcane *polyubiqitin* genes (Table II; Fig. 5) could be related to recovery from water stress caused by exposure to chilling temperatures.

One sugarcane EST encoding a putative XDH was significantly induced after 12 h of cold exposure (Table II). XDH is an NAD-dependent dehydrogenase

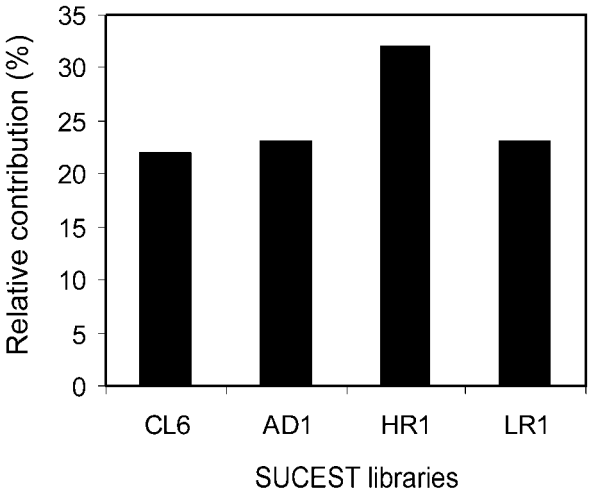

Figure 4. Relative contribution of the SUCEST libraries used in the macroarray experiments to identify cold-inducible genes. The values represent the percentage of normalized cold-inducible genes identified from each cDNA library (see "Results") in relation to the total number of ESTs identified as cold inducible in our arrays. CL6, Heat- and cold-treated and untreated callus; AD1, sugarcane plantlets infected with *Acetobacter diazotroficans*; HR1, plantlets infected with *Herbasperillum rubrisubalbicans*; and LR1, leaf row tissue.

that catalyzes the two final reactions of purine catabolism (Xu et al., 1996). This enzyme was induced by cold in fruitfly (*Drosophila melanogaster*; Duncker et al., 1995). Because chilling induces oxidative stress (Sato et al., 2001), it is possible that the sugarcane XDH is induced in response to oxidative stress generated by cold exposure. Another protein related to antioxidant metabolism is copper/zinc SOD (Cu/ZnSOD). This enzyme belongs to the group of metalloenzymes that protect cells from superoxide radicals by catalyzing the dismutation of the superoxide radical to molecular O_2 and H_2O_2 (Wu et al., 1999). A sugarcane EST encoding Cu/ZnSOD was induced after 24 h of cold exposure (Table II). The induction of two antioxidant genes suggests an increase in oxidative stress because cold-acclimating conditions do not result in the cessation of photosynthesis such that superoxide radicals were still being generated (Wu et al., 1999).

Kiyosue et al. (1998) observed that dehydration and chilling stress (4°C) induced a cDNA clone (ERD6) from Arabidopsis encoding a putative sugar transporter protein belonging to a multigene family (Williams et al., 2000). We also identified a sugarcane EST encoding a putative sugar transporter protein that was induced after 12 to 48 h of cold exposure (Table II). Sugar redistribution may be used as an energy source to protect cells against stress conditions (Kiyosue et al., 1998; Williams et al., 2000).

We also identified a cold-inducible inorganic phosphate transporter protein (Pht1-2). The role of nutrients such as nitrogen and phosphorus on cold hardiness has received attention because low temperatures inhibit photosynthesis and consequently reduce inorganic phosphate availability (Hurry et al., 2000). The induction of a sugarcane phosphate transporter gene may indicate a readjustment of the cellular P_i status and the recovery of photosynthetic carbon metabolism, thereby re-establishing the ability to produce Suc.

The recovery of photosynthetic carbon metabolism during cold stress may be achieved by increasing the

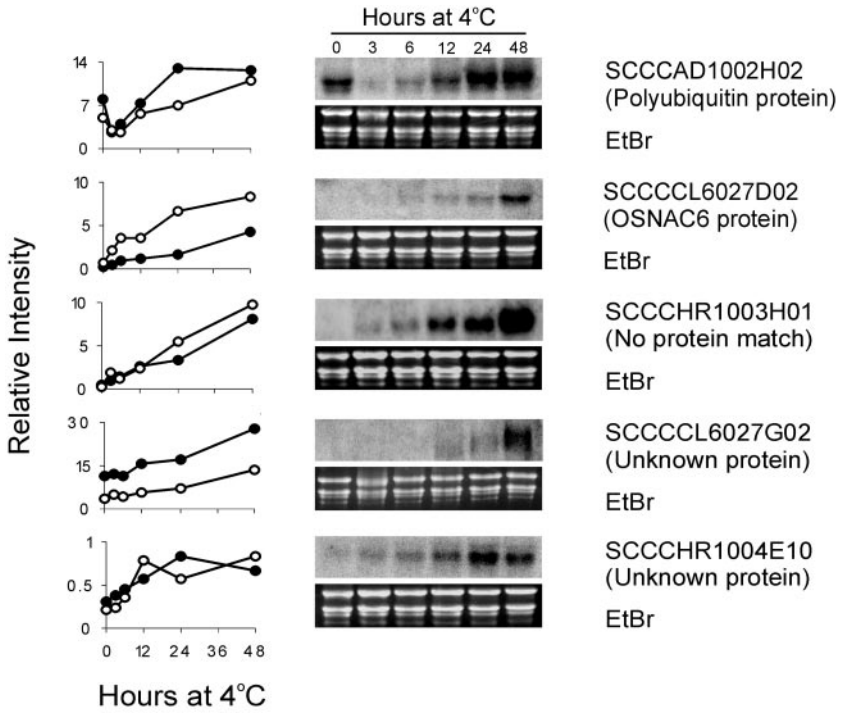

Figure 5. Comparison between EST macroarray and RNA-blot analysis for cold-inducible sugarcane ESTs. In RNA gel blots, each lane was loaded with 10 μg of total RNA isolated from plantlets grown at 26°C (0) and plantlets grown at 4°C for 3, 6, 12, 24, and 48 h. The graphs show the induction kinetics observed in the macroarrays (white circles) and RNA blots (black circles).

Table III. *Cold-regulated sugarcane homolog proteins identified by data mining the SUCEST database*

CHS, chalcone synthase; CLP, chitinase-like protein; P5CS, Δ (1) pyrroline-5-carboxylase synthetase; PLC1, phosphatidyl-specific phospholipase C protein; TLP, thaumatin-like protein; GLP, glucanase-like protein; ADH, alcohol dehydrogenase; GolS, galactinol synthase.

Assembled Sequences Identification[a]	Protein Similarity	Accession No.	Coverage[b]	E Value	Identity[d]	Similarity[d]	References
			%		%	%	
SCEPRZ1008C06	OsCDPK7	BAB16888	88	0.0	90	93	Saijo et al. (2000)
SCQSHR1023B08	CBF1	NP_567721	95	4E − 25	34	46	Stockinger et al. (2001)
SCBGLR1095B10	GCN5	O22929	80	4E − 34	57	72	Stockinger et al. (2001)
SCCCRZ1C01B03	ADA2b	AAK31320	99	1E − 137	49	59	Stockinger et al. (2001)
SCCCRZ1001D02	14-3-3	T04153	100	1E − 136	96	96	Jarillo et al. (1994)
SCRLLR1059D11	LIP15	S58692	100	6E − 48	70	74	Kusano et al. (1995)
SCCCRZ2001H12	NpCaM-1	P13565	100	1E − 80	100	100	van der Luit et al. (1999)
SCVPLR2027D06	CORTMC-AP3	CAA09867	100	3E − 37	52	62	Baldi et al. (1999)
SCCCLR1001F04	WCOR410b	T06802	100	2E − 27	33	39	Danyluk et al. (1998)
SCUTLR2015A11	WCOR413	AAG13395	96	2E − 89	82	86	Allard et al. (1998)
SCEQRT1028G10	CHS	AAD41878	99	0.0	96	96	Berberich et al. (1997)
SCJLRT1020F06	CLP	383024	99	1E − 143	75	80	Yu et al. (1999)
SCCCRZ1002G07	SUS1[c]	AAA68209	100	0.0	98	98	Déjardin et al. (1999)
SCEZRZ1016F07	α-Amylase	AAF63239	100	1E − 179	68	79	Wegrzyn et al. (2000)
SCACLR2014H05	β-Amylase	CAB58423	82	1E − 114	84	89	Seki et al. (2001)
SCCCRZ2C01H04	Ferritin	P29036	95	1E − 108	75	76	Seki et al. (2001)
SCQGLR1062E12	Glyoxalase I	BAA36759	100	1E − 149	88	92	Seki et al. (2001)
SCEPAM2011H12	atPUMP	CAA11757	96	1E − 127	73	79	Maia et al. (1998)
SCBFLR1026E05	V-ATPase	P49087	73	0.0	96	96	Carystinos et al. (1995)
SCCCRZ2C03E07	V-PPase[c]	S72527	94	0.0	96	97	Carystinos et al. (1995)
SCQGLR1085F11	DHN2	T14819	92	2E − 20	60	60	Zhu et al. (2000)
SCSGLR1045D05	HVA22	A48892	80	7E − 46	77	90	Shen et al. (2001)
SCCCLR1072E07	APX[c]	T03595	100	1E − 132	91	94	Zhang et al. (1997)
SCJLLR1054F05	HSC70	P24067	97	0.0	90	90	Li et al. (1999)
SCJFLR1073H12	P5CS	O04226	100	0.0	75	83	Gilmour et al. (2000)
SCSBHR1052C05	PLC1	AAK01711	75	0.0	71	77	Hirayama et al. (1995)
SCCCCL3003G05	60S Ribos. L13	BAA92738	94	2E − 95	86	89	Saez-Vasquez et al. (2000)
SCVPRT2073B04	TLP	444344	85	7E − 72	83	88	Yu et al. (1999)
SCJLRT1023E06	GLP	AF230109	91	3E − 75	49	65	Yu et al. (1999)
SCVPLR2005E09	ScRS7[c]	AAD26256	100	3E − 97	91	96	Berberich et al. (2000)
SCEZLR1031F04	GBF-1	T02084	97	1E − 162	78	80	de Vetten et al. (1995)
SCCCST2003C12	ADH	AAC34997	100	0.0	92	92	de Bruxelles et al. (1996)
SCCCLR1075C10	GolS	AAD26116	90	1E − 144	77	85	Pearce (1999)

[a]SUCEST-assembled sequence homologous to cold-regulated proteins reported for other plant species. [b]SUCEST-assembled sequence size relative to the total amino acid sequence of the driver protein. [c]Sugarcane protein homologs not been induced or repressed by cold treatment in our arrays. SUS1, Sucrose synthase 1; V-PPase, H^+-translocating pyrophosphatase; APX, ascorbate peroxidase; ScRS7, ribosomal protein S7. [d]The identity and similarity percentages were obtained using the BLOSUM62 matrix (http://www.ncbi.nlm.nih.gov).

activities of CO_2 fixation-related proteins such as PPDK and NADP-ME (Hurry et al., 2000). PPDK is an important enzyme in C_4 plant photosynthesis and is induced by ABA and stress associated with abiotic factors such as drying, cold, high salt, and mannitol treatment (Moons et al., 1998). NADP-ME is a major decarboxylating enzyme in NADP-ME-type C_4 species and has been reported to be induced by chilling stress (Fushimi et al., 1994). Sugarcane ESTs encoding PPDK and NADP-ME proteins were induced by cold exposition in our arrays (Table II), suggesting a possible maintenance of photosynthesis, even at low temperatures.

Members of the NAC protein family contain a highly conserved amino acid sequence in the N-terminal region known as the NAC domain (Kikuchi et al., 2000). The proteins of this family are specific to plant genomes and play important roles in plant growth, development, and senescence (Souer et al., 1996; Kikuchi et al., 2000). Recently, new members of the NAC family have been shown to interact with virus proteins and are involved in protein-protein interactions (Xie et al., 1999; Ren et al., 2000). Some NAC proteins also act as transcriptional regulators (Kikuchi et al., 2000). We identified three sugarcane ESTs encoding putative NAC proteins that were induced by cold exposure. Sequence analysis of these ESTs suggested that each one encodes a different member of the NAC family, with two of them encoding proteins similar to rice (*Oryza sativa*) NAC and the other one encoding a homolog of the Arabidopsis NAM protein (Table II). Interestingly, the ex-

Table IV. *Conserved domains of cold-inducible sugarcane putative and no hit proteins*

Clone Identification	Domain Description	Accession No.[a]	E Value	Similarity	Putative Function[b]
				%	
SCCCHR1004E10	SPX	pfam03105	2E − 22	48	G-protein-associated signal transduction; may also function as a phosphate sensor
SCEPCL6021A10	RING	smart00184	1E − 03	58	Protein-protein interactions
SCMCCL6027G02	DUF81	pfam01925	8E − 06	48	Unknown
SCEPCL6028F02	mTERF	pfam02536	2E − 17	57	DNA-binding motif (contains Leu zippers)
SCUTCL6036C05	e3-binding	pfam29152	4.6E − 08	40	Protein-protein interactions
SCCCHR1003H01	LEA 3	pfam03242	1.6E − 05	44	Desiccation tolerance
SCCCHR1003D12	Glycosylhydrolase	PF00332	5.1E − 23	76	Endo-1,3-β-glucanase-like protein

[a]Accession of each domain sequence was obtained by using the RPS-BLAST algorithm and the Pfam and SMART databases. [b]Putative functions expected based on similar domains described in SMART and Pfam domain family databases.

pression profiles of these ESTs differed from each other (Table II).

An EST encoding a protein similar to the OCSBF-1 was also induced by cold exposure (Table II). This protein was similar to the LIP19 of rice and LIP15 of maize (*Zea mays*), both of which are bZIP proteins up-regulated by exogenous ABA and low temperature (Singh et al., 1990; Aguan et al., 1991; Kusano et al., 1995). OCSBF-1 binds ocs cis-acting elements, G-box motifs, and ABA-responsive element (ABRE) in plant gene promoters (Kusano et al., 1995; Kim et al., 2001). Thus, sugarcane may have an ABA-dependent path-

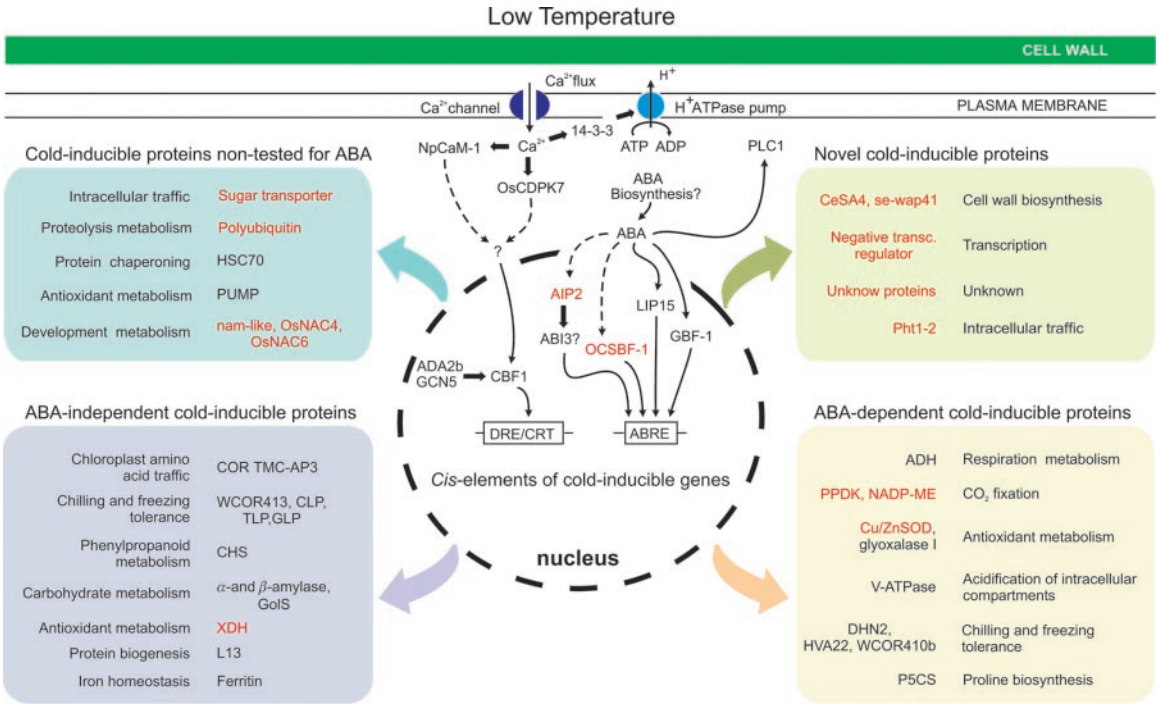

Figure 6. Hypothetical model of the sugarcane responses to low temperature. Colored rectangles contain pathways and protein names. The proteins identified by data mining are shown in black, whereas those in red were identified by macroarray expression profiling. Thin solid and dashed arrows represent gene induction (confirmed and putative, respectively) and large solid arrows represent protein interactions. OsCDPK7, rice Ca^{2+}-dependent protein kinase 7; NpCaM-1, calmodulin protein; 14-3-3, 14-3-3 protein; ADA2b, transcriptional adaptor; GCN5, HAT-like protein; CBF1, transcription factor containing AP2 DNA-binding motif; GBF-1, ABI3, LIP15, and OCSBF-1, bZIP transcription factors; AIP2,ABI3, interacting protein 2; COR TMC-AP3, chloroplastic amino acid selective channel protein; WCOR413, cold acclimation protein homolog F18B3.110; TLP, thaumatin-like protein; CLP, chitinase-like protein; GLP, glucanase-like protein; CHS, chalcone synthase; GolS, galactinol synthase; L13, ribosomal protein; Pht1-2, phosphate transporter; V-ATPase, vacuolar ATPase; HSC70, heat shock protein; PUMP, plant uncoupling mitochondrial protein; ADH, alcohol dehydrogenase; CeSA4, cellulose synthase 4; se-wap41, Golgi-associated protein; DHN2 and WCOR410b, dehydrin-like proteins; HVA22, ABA-inducible protein; P5CS, Δ (1) pyrroline-5-carboxylase synthetase; PLC1, phosphatidyl-specific phospholipase C protein; NAM-like, OsNAC4, and OsNAC6, NAC proteins. The cis-elements present in cold-inducible gene promoters are indicated by the rectangles: DRE/CRT and ABRE.

way triggered by low temperature. However, the presence of sugarcane protein similar to CBF1 (found in the data mining) also suggests the existence of an ABA-independent pathway (Table III; Fig. 6). These pathways may cross-talk during chilling stress.

Further evidence for the existence of an ABA-dependent cold-inducible pathway was the identification of a sugarcane EST encoding a protein similar to AIP2 from Arabidopsis. Significant induction of this gene was observed after 3 h of cold exposure (Table II). *AIP2* encodes a C_3HC_4-type zinc finger protein that interacts with ABA-INSENSITIVE3 (ABI3) protein during seed development in Arabidopsis (Kurup et al., 2000). ABI3 also appears to modulate low-temperature-induced freezing tolerance (Tamminen et al., 2001). Although no SUCEST-assembled sequence similar to ABI3 protein was identified, we cannot exclude the possibility that the sugarcane genome encodes this protein.

A sugarcane EST encoding a protein similar to an Arabidopsis general negative transcription regulator was up-regulated after 48 h of cold exposure (Table II). To our knowledge, this is the first time that the induction of a negative transcription regulator has been associated with low temperatures. This protein may be involved in down-regulating specific genes in response to chilling stress.

Finally, we identified two cold-inducible sugarcane ESTs encoding proteins similar to cellulose synthase 4 (CeSA4) and se-wap41. CeSA4 is involved in the production of cellulose, the major component of all higher plant cell walls (Richmond, 2000). Se-wap41 is a reversible glycosylated polypeptide associated with the Golgi complex that is involved in the synthesis of xyloglucan and other hemicelluloses (Dhugga et al., 1997). Nevertheless, the role of this putative reinforcement of the sugarcane leaf cell wall by cellulose and other components is not evident in chilling stress, because it has been suggested to function as a barrier against ice propagation during freezing stress (Pearce, 1999).

The repression of gene expression may also be an important component of the adaptation to low temperatures. To our knowledge, most of the ESTs found in our experiments represented genes that have not previously been reported as being down-regulated by chilling or freezing stresses. However, an aquaporin gene (*rwc1*) was identified as being chilling-repressed in rice leaves (Li et al., 2000). The authors suggested that this gene is involved in responses to water stress induced by chilling. It is possible that reduced transcript levels of sugarcane aquaporin (Table II) keep a suitable status of intracellular water during chilling stress.

Cold-Inducible Sugarcane Genes Mined in SUCEST Database and a Putative Model for Sugarcane Response to Low Temperature

A database containing the sequences of cold-inducible genes identified in different plant species was used as a driver to identify putative homologs in the SUCEST database. Thirty-three sugarcane-assembled sequences were identified, of which four were also found in our arrays (Table III). These genes are involved in many different functions including signaling pathways, transcriptional regulation, and other metabolic processes. The information associated with these proteins together with that provided by our arrays was used to develop a model for sugarcane responses to cold (Fig. 6).

A transient Ca^{2+} influx through plasma membrane Ca^{2+} channels or Ca^{2+} release from vacuole occurs during the initiation of cold acclimation (Monroy and Dhindsa, 1995; Knight et al., 1996; Knight and Knight, 2000). In addition, some Ca^{2+}-dependent proteins may be induced by low temperatures in plants (Jarillo et al., 1994; Monroy and Dhindsa, 1995; van der Luit et al., 1999). In our proposed model for sugarcane responses to cold adaptation (Fig. 6), the transient Ca^{2+} influx could be sufficient to activate Ca^{2+}-dependent multifunctional proteins such as 14-3-3, Ca^{2+}-dependent protein kinase (OsCDPK7), and calmodulin (NpCaM-1).

One SUCEST-assembled sequence showed similarity to CBF1 protein (Table III), including the nearly identical signature sequences (PPK/RPAGRxKFx-ETRHP and DSAWL) surrounding the AP2/EREBP domain, characteristic of CBF proteins (Jaglo et al., 2001). Gilmour et al. (1998) suggested that *CBF1* encodes the AP2-like transcriptional activator that binds to the CRT/DRE regulatory element present in the promoter of many *COR* genes and stimulates their transcription. On the basis of these observations, we speculate that sugarcane has cold-inducible genes that are members of the CBF regulon. While we were preparing this paper, Fowler and Thomashow (2002) reported that cold acclimation induced the expression of novel members of the CBF regulon in Arabidopsis. Among them, there are genes encoding for a putative sugar transporter and a galactinol synthase protein that were up-regulated by cold in our arrays and were also found in the SUCEST data mining (Tables II and III).

Another recent fundamental advance in understanding cold-associated transcriptional control mechanisms was the discovery of Arabidopsis histone acetyltransferase (HAT)-containing adapter complexes, which are recruited to promoters by transcriptional factors. These proteins can stimulate transcription (Stockinger et al., 2001). These complexes consist of the proteins GCN5 and ADA (Grant et al., 1997). The GCN5 protein has HAT activity, whereas the ADA protein may function as a transcriptional adapter for some activation domains (Marcus et al., 1994; Stockinger et al., 2001). We identified SUCEST-assembled sequences similar to Arabidopsis GCN5 and ADA2b proteins (Table III). These results provide preliminary evidence for the existence of HAT-

containing adapter complexes in sugarcane. The existence of this complex in plants was believed to be restricted to Arabidopsis (Stockinger et al., 2001). As in Arabidopsis (Stockinger et al., 2001), sugarcane GCN5 and ADA2b proteins may interact physically with each other and with CBF1, thereby stimulating the transcription of CBF1 target genes (Fig. 6).

We found assembled sequences encoding bZIP transcription factors similar to the G-box-binding factor 1 (GBF-1) and LIP15 (de Vetten and Ferl, 1995; Kusano et al., 1995; Kim et al., 2001), which bind to the ABRE cis-element present in cold- and ABA-induced gene promoters. Kim et al. (2001) observed that the level of *SGBF-1* transcripts in soybean (*Glycine max*) was up-regulated by cold and ABA. On the basis of these data, the LIP15 and GBF-1 proteins in sugarcane may be induced by cold treatment and may bind to the ABRE motif, thus enhancing the expression of cold-inducible genes through a cold-induced ABA-dependent pathway (Fig. 6).

A sugarcane-assembled sequence homologous to phosphatidyl-specific phospholipase C protein from Arabidopsis (atPLC-1; Hirayama et al., 1995) was also identified in the SUCEST database (Table III). This enzyme, which is induced by low temperature and ABA, hydrolyzes phosphatidylinositol 4,5-biphosphate into the 2-s messengers 1,4,5-triphosphate and 1,2-diacylglycerol (Hirayama et al., 1995).

Mining of the SUCEST database allowed us to identify several other proteins known to be induced by cold exposure (Table III). For instance, Δ (1) pyrroline-5-carboxylase synthetase, galactinol synthase, and α- and β-amylases may contribute to solute accumulation (Pearce, 1999; Gilmour et al., 2000; Seki et al., 2001). Other proteins, such as plant uncoupling mitochondrial protein, which can decrease the generation of reactive oxygen species during chilling stress (Maia et al., 1998), were also identified.

The cold anaerobic conditions caused by waterlogging and cold-induced increase of endogenous ABA can up-regulate alcohol dehydrogenase expression (de Bruxelles et al., 1996). In addition, other pathways, such as phenylpropanoid biosynthesis, may be involved in reactive oxygen species scavenging (Berberich and Kusano, 1997). We found an assembled sequence encoding a protein similar to chalcone synthase that catalyzes the first step in the branch pathway of phenylpropanoid synthesis specific for the formation of flavonoid products, which can function as antioxidant compounds (Grace and Logan, 2000). We also found an assembled sequence encoding a protein similar to the chloroplast amino acid-selective channel protein (COR TMC-AP3) from barley (*Hordeum vulgare*), whose gene expression is up-regulated by cold (Baldi et al., 1999).

A putative up-regulation of HSC70 mRNA expression in sugarcane during chilling stress may be required to sustain high levels of this heat shock protein that would stabilize some proteins compromised at low, nonfreezing temperatures (Li et al., 1999). Sugarcane protein biogenesis can also be altered during cold exposure through induction of a 60S ribosomal L13-like protein (Sáez-Vásquez et al., 2000).

Finally, we found ESTs encoding proteins that have been shown to be directly involved in chilling and freezing tolerance, including WCOR410b (Danyluk et al., 1998), WCOR413 (Allard et al., 1998), dehydrin 2 (DHN2; Zhu et al., 2000), barley ABA-inducible protein (HVA22; Shen et al., 2001), thaumatin-like protein, glucanase-like protein, and chitinase-like protein (Yu and Griffith, 1999). Our computer analyses suggest that sugarcane has two putative dehydrin-like proteins (WCOR410b and DHN2). These proteins could stabilize macromolecules and/or protect membranes against chilling damage (Pearce, 1999). Moreover, thaumatin-like protein, glucanase-like protein, and chitinase like-protein are examples of pathogenesis-related proteins with antifreeze activity, which can have direct effects on the stability of cellular membranes (Hincha et al., 1997; Pearce, 1999). A β-1,3-glucanase, for instance, has been reported as a protecting factor of thylakoid membranes against freeze-thaw effects (Hincha et al., 1997), as well as a fish antifreeze protein type I, which inhibited leakage across membranes during chilling to nonfreezing temperatures (Tomczak et al., 2002). It is possible that sugarcane putative antifreeze proteins can confer cellular membrane protection, reducing chilling injury.

MATERIALS AND METHODS

Plant Growth and Cold Treatment

Sugarcane (*Saccharum* sp. cv SP80-3280) plantlets were propagated axenically in vitro by excising the shoot apex of 2-month-old sugarcane plants kept in a greenhouse and culturing them in 5 mL of Murashige and Skoog medium (Murashige and Skoog, 1962) containing 150 mg citric acid L^{-1}. After 1 month, the shoots were transferred to 50 mL of the same medium supplemented with 0.2 mg 6-benzylaminopurine L^{-1} and 0.1 mg kinetin L^{-1}. Proliferating plantlets were subcultured every 3 weeks. Plantlets were then transferred to rooting medium in which 6-benzylaminopurine was substituted by 0.2 mg indole butyric acid L^{-1}. All plantlets were kept in a growth chamber at 26°C on a 16-h/8-h day/night cycle with a photon flux density of 70 μE m^{-2} s^{-1}. After 4 weeks, when the roots were fully developed, the plantlets were transferred to a growth chamber under the same photoperiod conditions but at 4°C. Control plantlets were maintained at 26°C. The leaves of control and cold-treated plantlets were harvested after 0, 3, 6, 12, 24, and 48 h of treatment. Six plantlets were used for each time point.

High-Density Filter Arrays and Probe Preparation

Sixteen 96-well plates containing EST plasmid clones were randomly sampled from the following sugarcane cDNA libraries: heat- and cold-treated and untreated callus (CL6), sugarcane plantlets infected with *H. rubrisubalbicans* (HR1) or *A. diazotroficans* (AD1), and leaf row tissue (LR1; Vettore et al., 2001). The plasmid DNA was denatured in 0.2 N NaOH for 15 min at 37°C and then spotted onto Hybond-N filters (Amersham Biosciences, Piscataway, NJ) with a hand-held 96-pin printhead tool (V&P Scientific, San Diego, CA). This tool typically deposited 0.1 μL of DNA solution, which corresponded to approximately 5 ng of DNA. The set of sixteen 96-well plates was spotted on two nylon filters (85 × 125 mm) in a 4 × 4 array configuration (768 ESTs per filter). Additionally, 12 spots

containing DNA of the empty plasmid vector pSPORT1 (Invitrogen, Carlsbad, CA) were applied to the filters as a negative hybridization control. The DNA was fixed to the filters by baking at 80°C for 2 h, and the filters were then stored at room temperature until used for hybridizations.

Total RNA was isolated from the leaves of treated and untreated sugarcane plantlets using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Probes were produced as described by Schummer et al. (1999) with slight modifications. In brief, 30 μg of total RNA was reverse transcribed with Superscript II (Invitrogen) using an oligo-dT18V (3 μM) primer, with 3,000 Ci mmol^{-1} [α-^{33}P]dCTP and unlabeled dATP, dGTP, and dTTP (1 mM each) for 20 min at 42°C. Unlabeled dCTP was then added to a final concentration of 1 mM, and the reaction continued for another 40 min. The cDNA probes were purified by using ProbeQuant G-50 microcolumns according to the manufacturer's instructions (Amersham Biosciences). Variations in the amount of DNA in the spots were estimated by hybridizing the filters with an oligonucleotide probe that recognized the sequence of the *Amp*^{r} gene of the pSPORT1 vector. This probe was synthesized with the primers 5′-GTGGTCCTGCAACTTTATCCGC-3′ and 5′-TAGACTGGATGGAGGCGGATAA-3′ in the presence of [α-^{33}P]dCTP, according to the protocol described by McPherson (2000; http://www.tree.caltech.edu/protocols/overgo.html).

Macroarray Analysis

Filters were initially hybridized with the oligo vector probe for 16 h at 58°C. Further details of the hybridization procedures can be obtained from the Web site cited in the previous paragraph. After hybridization and washing, the filters were exposed to imaging plates for 96 h and then scanned in a phosphorimager FLA3000-G (Fujifilm, Tokyo). The oligo vector probe was removed from the filters by boiling in 0.1% (w/v) SDS solution, with the efficiency of probe removal being monitored by phosphorimager scanning. After stripping, the filters were hybridized with cDNA probes for 18 h at 42°C as described by Schummer et al. (1997) and then sequentially washed in the following solutions: 0.2× SSC with 0.5% (w/v) SDS for 20 min at room temperature, 0.5× SSC with 0.5% (w/v) SDS for 20 min at 65°C (twice), and 0.2× SSC with 0.5% (w/v) SDS for 20 min at 65°C. After the last wash, the filters were sealed with plastic film, immediately exposed to imaging plates for 96 h, and scanned as described above. All signals were quantified by using Array Vision software (Imaging Research, St. Catherines, ON, Canada). The grids were predefined and manually adjusted to obtain optimal spot recognition, and the spots were then quantified individually. The local background was subtracted automatically from each spot, and the intensity data were rearranged into Microsoft Excel (Microsoft, Redmond, WA) files for further analysis. The two signal intensity values of duplicated DNA spots were averaged and used to calculate the expression ratios between cold-treated and control (untreated) samples.

RNA-Blot Analysis

Ten micrograms of total RNA was electrophoresed in a 1% (w/v) agarose gel containing formaldehyde and transferred to a Hybond-N+ filter (Amersham Biosciences) as described by Sambrook et al. (1989). The filters were hybridized with the cDNA inserts of cold-inducible sugarcane ESTs labeled with [α-^{32}P]dCTP, and hybridization was done at 42°C (Sambrook et al., 1989). The blots were then washed at high stringency and exposed to imaging plates. Digitized images of the RNA-blot hybridization signals were quantified using the Image Gauge software (Fujifilm).

Bioinformatics

The sequences of 250 cold-related plant proteins obtained from the National Center for Biotechnology Information were analyzed for similarity against the 43,141 assembled sequences of the SUCEST database (Telles and da Silva, 2001) using the tBLASTN algorithm. Unknown protein domain analysis was done using the RPS-BLAST algorithm, Pfam (Bateman et al., 2000), and SMART (Schultz et al., 2000) databases.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Accession Numbers

The sequence data described in this paper have been submitted to GenBank under accession numbers BU102492 to BU103710. The array data described in this manuscript have been submitted to Gene Expression Omnibus under accession numbers GPL210 (platform), GSM2431 to GSM2442 (samples), and GSE83 and GSE84 (series).

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LITERATURE CITED

Aguan K, Sugawara K, Suzuki N, Kusano T (1991) Isolation of genes for low-temperature-induced proteins in rice by a simple subtractive method. Plant Cell Physiol **32:** 1285–1289

Allard F, Houde M, Kr M, Ivanov A, Huner NPA, Sarhan F (1998) Betaine improves freezing tolerance in wheat. Plant Cell Physiol **39:** 1194–1202

Allen DJ, Ort DR (2001) Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci **6:** 36–42

Baldi P, Grossi M, Pecchioni N, Valè G, Cattivelli L (1999) High expression level of a gene coding for a chloroplastic amino acid selective channel protein is correlated to cold acclimation in cereals. Plant Mol Biol **41:** 233–243

Bateman A, Birney E, Durbin R, Eddy SR, Howe KL, Sonnhammer ELL (2000) The Pfam protein families database. Nucleic Acids Res **28:** 263–266

Berberich T, Kusano T (1997) Cycloheximide induces a subset of low temperature-inducible genes in maize. Mol Gen Genet **254:** 275–283

Berberich T, Uebeler M, Feierabend J (2000) cDNA cloning of cytoplasmic ribosomal protein S7 of winter rye (*Secale cereale*) and its expression in low-temperature-treated leaves. Biochim Biophys Acta **1492:** 276–279

Carystinos GD, MacDonald HR, Monroy AF, Dhindsa RS, Poole RJ (1995) Vacuolar H^+-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. Plant Physiol **108:** 641–649

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol **3:** 117–124

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell **10:** 623–638

de Bruxelles GL, Peacock WJ, Dennis ES, Dolferus R (1996) Abscisic acid induces the alcohol dehydrogenase gene in Arabidopsis. Plant Physiol **111:** 381–391

Déjardin A, Sokolov LN, Kleczkowski LA (1999) Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. Biochem J **344:** 503–509

de Vetten NC, Ferl RJ (1995) Characterization of a maize G-box binding factor that is induced by hypoxia. Plant J **7:** 589–601

Dhugga KS, Tiwari SC, Ray PM (1997) A reversibly glycosylated polypeptide (RGP1) possibly involved in plant cell wall synthesis: purification, gene cloning, and trans-Golgi localization. Proc Natl Acad Sci USA **94:** 7679–7684

Du Y-C, Nose A, Wasano K (1999) Thermal characteristics of C4 photosynthetic enzymes from leaves of three sugarcane species differing in cold sensitivity. Plant Cell Physiol **40:** 298–304

Duncker BP, Chen C-P, Davies PL, Walker VK (1995) Antifreeze protein does not confer cold tolerance to transgenic *Drosophila melanogaster*. Cryobiology **32:** 521–527

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold

acclimation in addition to the CBF cold response pathway. Plant Cell **14:** 1675–1690

Freeman WM, Robertson DJ, Vrana KE (2000) Fundamentals of DNA hybridization arrays for gene expression analysis. Biotechnology **29:** 1042–1055

Friddle CJ, Koga T, Rubin EM, Bristow J (2000) Expression profiling reveals distinct sets of genes altered during induction and regression of cardiac hypertrophy. Proc Natl Acad Sci USA **97:** 6745–6750

Fushimi T, Umeda M, Shimazaki T, Kato A, Toriyama K, Uchimiya H (1994) Nucleotide sequence of a rice cDNA similar to a maize NADP-dependent malic enzyme. Plant Mol Biol **24:** 965–967

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854–1865

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-inducible *COR* gene expression. Plant J **16:** 433–442

Girke T, Todd J, Ruuska S, White J, Benning C, Ohlrogge J (2000) Microarray analysis of developing Arabidopsis seeds. Plant Physiol **124:** 1570–1581

Grace SC, Logan BA (2000) Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philos Trans R Soc Lond B Biol Sci **355:** 1499–1510

Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba R, Owen-Hughes T, Allis CD, Winston F et al. (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev **11:** 1640–1650

Guy CL, Niemi KJ, Brambi R (1985) Altered gene expression during cold acclimation of spinach. Proc Natl Acad Sci USA **82:** 3673–3677

Hincha DK, Meins F Jr, Schmitt JM (1997) β-1,3-Glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation. Plant Physiol **114:** 1077–1083

Hirayama T, Ohto C, Mizoguchi T, Shinozaki K (1995) A gene encoding a phosphatidyl-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **92:** 3903–3907

Hurry V, Strand A, Furbank R, Stitt M (2000) The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the *pho* mutants of *Arabidopsis thaliana*. Plant J **24:** 383–396

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol **127:** 910–917

Jarillo JA, Capel J, Leyva A, Martinez-Zapater JM, Salinas J (1994) Two related low-temperature-inducible genes of Arabidopsis encode proteins showing high homology to 14-3-3 proteins, a family of putative kinase regulators. Plant Mol Biol **25:** 693–704

Kikuchi K, Ueguchi-Tanaka M, Yoshida KT, Nagato Y, Matsusoka M, Hirano H-Y (2000) Molecular analysis of the *NAC* gene family in rice. Mol Gen Genet **262:** 1047–1051

Kim JC, Lee SH, Cheong YH, Yoo C-M, Lee SI, Chun HJ, Yun D-J, Hong JC, Lee SY, Lim CO et al. (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J **25:** 247–259

Kiyosue T, Abe H, Yamaguchi-Shinozaki K, Shinozaki K (1998) ERD6, a cDNA clone for an early dehydration-induced gene of Arabidopsis, encodes a putative sugar transporter. Biochim Biophys Acta **1370:** 187–191

Knight H, Knight MR (2000) Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in Arabidopsis. J Exp Bot **51:** 1679–1686

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489–503

Kurup S, Jones HD, Holdsworth MJ (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J **21:** 143–155

Kusano T, Berberich T, Harada M, Suzuki N, Sugawara K (1995) A maize DNA-binding factor with a bZIP motif is induced by low temperature. Mol Gen Genet **248:** 507–517

Li L-G, Li S-F, Tao Y, Kitagawa Y (2000) Molecular cloning of a novel water channel from rice: its products expression in *Xenopus* oocytes and involvement in chilling tolerance. Plant Sci **154:** 43–51

Li Q-B, Haskell DW, Guy CL (1999) Coordinate and non-coordinate expression of the stress 70 family and other molecular chaperones at high and low temperature in spinach and tomato. Plant Mol Biol **39:** 21–34

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcriptions factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391–1406

Maia IG, Benedetti CE, Leite A, Turcinelli SR, Vercesi AE, Arruda P (1998) AtPUMP: an Arabidopsis gene encoding a plant uncoupling mitochondrial protein. FEBS Lett **429:** 403–406

Marcus GA, Silverman N, Berger SL, Horiuchi J, Guarente L (1994) Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. EMBO J **13:** 4807–4815

McPherson JD (2000) Overgo probing of high-density filters. http://www.tree.caltech.edu/protocols/overgo.html

Medina J, Bargues M, Terol J, Pérez-Alonso M, Salinas J (1999) The *Arabidopsis CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol **119:** 463–469

Monroy AF, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell **7:** 321–331

Moons A, Valcke R, Van Montagu M (1998) Low-oxygen stress and water deficit induce cytosolic pyruvate orthophosphate dikinase (PPDK) expression in roots of rice, a C3 plant. Plant J **15:** 89–98

Murashige T, Skoog F (1962) A revised medium for rapid growth and biossays with tobacco tissue culture. Physiol Plant **15:** 471–497

O'Mahony PJ, Oliver MJ (1999) The involvement of ubiquitin in vegetative desiccation tolerance. Plant Mol Biol **41:** 657–667

Pearce RS (1999) Molecular analysis of acclimation to cold. Plant Growth Regul **29:** 47–76

Perret E, Ferrán EA, Marinx O, Liauzun P, Dumont X, Fournier J, Kaghad M, Ferrara P, Caput D (1998) Improved differential screening approach to analyze transcription variations in organized cDNA libraries. Gene **208:** 103–115

Ren T, Qu F, Morris TJ (2000) *HRT* gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell **12:** 1917–1925

Richmond T (2000) Higher plant cellulose synthases. Genome Biol **1:** 3001.1–3001.6

Rose D (2000) Microfluidic technologies and instrumentation for printing DNA microarrays. *In* M Schena, eds, Microarray Biochip Technology, Ed 1. Eaton Publishing, BioTechniques Books Division, Natick, MA, pp 19–37

Sáez-Vásquez J, Gallois P, Delseny M (2000) Accumulation and nuclear targeting of BnC24, a *Brassica napus* ribosomal protein corresponding to a mRNA accumulating in response to cold treatment. Plant Sci **156:** 35–46

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J **23:** 319–327

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sato Y, Murakami T, Funatsuki H, Matsuba S, Saruyama H, Tanida M (2001) Heat shock-mediated APX gene expression and protection against chilling injury in rice seedlings. J Exp Bot **51:** 1679–1686

Schena M, Heller RA, Theriault TP, Konrad K, Lachenmeier E, Davis RW (1998) Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol **16:** 301–306

Schultz J, Copley RR, Doerks T, Ponting CP, Bork P (2000) SMART: a web-based tool for the study of genetically mobile domains. Nucleic Acids Res **28:** 231–234

Schummer M, Ng W-L, Bumgarner RE, Nelson PS, Schummer B, Bednarski DW, Hassell L, Baldwin RI, Karlan BY, Hood L (1999) Comparative

hybridization of an array of 21500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. Gene **238:** 375–385

Schummer M, Ng W-L, Nelson PS, Bumgarner RB, Hood L (1997) A simple high-performance DNA arraying device for comparative expression analysis of a large number of genes. Biotechnology **23:** 1087–1092

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1,300 Arabidopsis genes under drought and cold stress by using a full-length cDNA microarray. Plant Cell **13:** 61–72

Shen Q, Chen CN, Brands A, Pan SM, Ho TH (2001) The stress- and abscisic acid-induced barley gene HVA22: developmental regulation and homologues in diverse organisms. Plant Mol Biol **45:** 327–340

Singh K, Dennis ES, Ellis JG, Llewellyn DJ, Tokuhisa JG, Wahleithner JA, Peacock WJ (1990) OCSBF-1, a maize ocs enhancer binding factor: isolation and expression during development. Plant Cell **2:** 891–903

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The *no apical meristem* gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell **85:** 159–170

Stockinger EJ, Mao Y, Regier MK, Triezenberg SJ, Thomashow MF (2001) Transcriptional adaptor and histone acetyltransferase proteins in Arabidopsis and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. Nucleic Acids Res **29:** 1524–1533

Tai PYP, Lentini RS (1998) Freeze damage of Florida sugarcane. *In* DL Anderson, eds, Sugarcane Handbook, Ed 1. Florida Cooperative Extension Service, University of Florida, Gainesville, FL, pp 1–3

Tamminen I, Makela P, Heino P, Palva ET (2001) Ectopic expression of ABI3 gene enhances freezing tolerance in response to abscisic acid and low temperature in *Arabidopsis thaliana*. Plant J **25:** 1–8

Telles GP, da Silva FR (2001) Trimming and clustering sugarcane ESTs. Gen Mol Biol **24:** 17–23

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol **118:** 1–7

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol **125:** 89–93

Tomczak MM, Hincha DK, Estrada SD, Wolkers WF, Crowe LM, Feeney RE, Tablin F, Crowe JH (2002) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. Biophys J **82:** 874–881

van der Luit A, Olivari C, Haley A, Knight MR, Trewavas AJ (1999) Distinct calcium signaling pathways regulate calmodulin gene expression in tobacco. Plant Physiol **121:** 705–714

Vettore A, da Silva FR, Kemper EL, Arruda P (2001) The libraries that made SUCEST. Gen Mol Biol **24:** 1–7

Wegrzyn T, Reilly K, Cipriani G, Murphy P, Newcomb R, Gardner R, MacRae E (2000) A novel alpha-amylase gene is transiently up-regulated during low temperature exposure in apple fruit. Eur J Biochem **267:** 1313–1322

Williams LE, Lemoine R, Sauer N (2000) Sugar transporters in higher plants: a diversity of roles and complex regulation. Trends Plant Sci **5:** 283–290

Wu G, Wilen RW, Robertson AJ, Gusta LV (1999) Isolation, chromosomal localization, and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat. Plant Physiol **120:** 513–520

Xie Q, Sanz-Burgos AP, Guo H, Gárcia JA, Gutiérrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol **39:** 647–656

Xin Z, Browse J (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. Plant Cell Environ **23:** 893–902

Xu P, Huecksteadt TP, Hoidal JR (1996) Molecular cloning and characterization of the human xanthine dehydrogenase gene (XDH). Genomics **34:** 173–180

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264

Yu XM, Griffith M (1999) Antifreeze proteins in winter rye leaves form oligomeric complexes. Plant Physiol **119:** 1361–1370

Zhang H, Wang J, Nickel U, Allen RD, Goodman HM (1997) Cloning and expression of an Arabidopsis gene encoding a putative peroxisomal ascorbate peroxidase. Plant Mol Biol **34:** 967–971

Zhu B, Choi DW, Fenton R, Close TJ (2000) Expression of the barley dehydrin multigene family and the development of freezing tolerance. Mol Gen Genet **264:** 145–153