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Gene Expression Phenotypes of Arabidopsis Associated with Sensitivity to Low Temperatures[w]

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Chilling is a common abiotic stress that leads to economic losses in agriculture. By comparing the transcriptome of Arabidopsis under normal (22°C) and chilling (13°C) conditions, we have surveyed the molecular responses of a chilling-resistant plant to acclimate to a moderate reduction in temperature. The mRNA accumulation of approximately 20% of the approximately 8,000 genes analyzed was affected by chilling. In particular, a highly significant number of genes involved in protein biosynthesis displayed an increase in transcript abundance. We have analyzed the molecular phenotypes of 12 chilling-sensitive mutants exposed to 13°C before any visible phenotype could be detected. The number and pattern of expression of chilling-responsive genes in the mutants were consistent with their final degree of chilling injury. The mRNA accumulation profiles for the chilling-lethal mutants *chs1*, *chs2*, and *chs3* were highly similar and included extensive chilling-induced and mutant-specific alterations in gene expression. The expression pattern of the mutants upon chilling suggests that the normal function of the mutated loci prevents a damaging widespread effect of chilling on transcriptional regulation. In addition, we have identified 634 chilling-responsive genes with aberrant expression in all of the chilling-lethal mutants. This reference gene list, including genes related to lipid metabolism, chloroplast function, carbohydrate metabolism and free radical detoxification, represents a potential source for genes with a critical role in plant acclimation to suboptimal temperatures. The comparison of transcriptome profiles after transfer of Arabidopsis plants from 22°C to 13°C versus transfer to 4°C suggests that quantitative and temporal differences exist between these molecular responses.

Chilling and cold, referring to low but not freezing temperatures frequently occurring in nature, damage many species of plants, especially those of tropical origin, by causing wilting, chlorosis, or necrosis, thus restricting their growth and development (Lyons, 1973; Wang, 1990). In cold-sensitive plants, physiological alterations such as leakage of ions from cell membranes and changes in photosynthesis and respiration occur due to exposure to low temperatures (Lyons, 1973; Huner, 1988). Besides general responses to acclimate their physiology to the temperature change, cold-acclimation responses are induced by low but nonfreezing temperatures to enhance freezing tolerance (Levitt, 1980). Adaptative mechanisms occurring during cold acclimation include accumulation of Pro and other cryoprotectants, changes in lipid membrane composition, alterations in photosynthetic carbon metabolism and detoxification of active oxygen species (AOS; Iba, 2002, and refs. therein; Stitt and Hurry, 2002).

To investigate cold acclimation to freezing temperatures, numerous molecular genetic studies have been performed using Arabidopsis, a freezing-resistant plant, as a model system. These approaches have resulted in the identification of a regulatory network with downstream genes induced by cold treatments (4°C, we will refer to this temperature as cold treatment here; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001; Fowler and Thomashow, 2002). Many of these genes are also induced by abscisic acid or by dehydration, which is consistent with the fact that abscisic acid and dehydration can increase freezing tolerance (Mantyla et al., 1995). Among cold-regulated genes, the C-repeat binding factor (*CBF*) genes, regulators for the cold-induced transcriptional cascade, have been shown to increase freezing tolerance when overexpressed in transgenic plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998). However, the existence of CBF-parallel pathways involved in cold-acclimation has been supported by transcription profiling of plants overexpressing the three members of the *CBF* family (Fowler and Thomashow, 2002) and the Arabidopsis mutant *eskimo1*, which displays freezing tolerance in the absence of cold treatments without changes in expression of the components of the CBF-pathway (Xin and Browse, 1998). In contrast to cold-acclimation and freezing tolerance, much less is known about molecular changes affecting regulatory and biochemical mech-

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anisms triggered to optimize growth at low but above-freezing temperatures. Moreover, it is currently unknown whether molecular mechanisms for cold resistance and acclimation to chilling temperatures (such as 13°C, referred to as chilling in this study) in Arabidopsis are related.

Chilling, cold, and freezing resistance in plants are believed to be at least partly mediated by changes in the biophysical properties of cellular membranes affecting the transition temperature from the gel (solid) to the liquid-crystalline phase (Miquel et al., 1993; Gibson et al., 1994; Steponkus et al., 1998). The fluidity of membranes at low temperature depends on the extent of lipid desaturation and can affect the activity of membrane-bound enzymes (Houslay and Gordon, 1984). Several experiments have indicated that alterations in the amount of polyunsaturated fatty acids in the fatty acid component of phosphatidylglycerol modify a plant's sensitivity to temperature changes (for review, see Browse and Somerville, 1994). In plants, the first committed step toward synthesis of the predominant C18-unsaturated fatty acids is catalyzed by a stearoyl-ACP desaturase in plastids. Acyl-ACPs are then esterified into glycerolipids by glycerol-3-phosphate acyltransferase. In Arabidopsis, increased cold-sensitivity has been triggered by alterations in the fatty acid composition of plastid membranes by overexpression of a glycerol-3-phosphate acyltransferase encoded by the *Escherichia coli pls*B gene, which is selective for saturated acyl-ACP substrates (Wolter et al., 1992). Further desaturation is mediated by acyl-lipid desaturases, which use glycerolipids as substrates. Supporting the importance of acyl-lipid desaturases, changes in sensitivity to low temperatures in tobacco (*Nicotiana tabacum*) were observed by overexpressing *FAD7*, a chloroplast-localized ω3-desaturase gene from Arabidopsis (Kodama et al., 1994), and a Δ9-desaturase gene from cyanobacteria with broad specificity (Ishizaki-Nishizawa et al., 1996). In Arabidopsis, desaturase mutants *fad5* and *fad6* become chlorotic after they are kept at 6°C (Hugly and Somerville, 1992), and the transcript of the *FAD8* desaturase gene accumulates upon low temperature treatments (Gibson et al., 1994). Similarly, two desaturases have been identified in maize (*Zea mays*) that exhibit different mRNA accumulation under normal and low temperatures (Berberich et al., 1998). Recently, it has been shown that the reduced expression of a putative aminophospholipid translocase results in cold-affected plants that are much smaller than the wild type (Gomès et al., 2000).

Protein metabolism is another cellular function known to be affected by exposure to low temperature in several organisms (Fujita, 1999; Los and Murata, 1999). In plants, less is know about temperature-regulated changes in protein synthesis. However, it has been reported that chloroplast protein accumulation is reduced in several chilling-sensitive mutants of Arabidopsis (Schneider et al., 1995). In addition, cold-induced heat shock proteins have been proposed to function as chaperonins to aid in correct protein biogenesis during cold exposure (Anderson et al., 1994).

A simple approach to identify molecular components that might be required for plant acclimation to suboptimal temperatures is the molecular analysis of mutants with increased sensitivity to chilling. To date, more than 20 Arabidopsis mutants with this phenotype have been identified (Schneider et al., 1994). These mutants have been characterized and sorted into classes based on genetic analyses and on the visible phenotypes they display 1 week after chilling treatments (Schneider et al., 1994). The most severe phenotypes were observed for class 1 mutants: *chs1* and *chs3* mutants cease to grow when shifted to 18°C or below, and *chs2* mutants cease to grow at 14°C or below (Schneider et al., 1994). Moreover, after 3 d of chilling, these mutations become lethal, and none of these mutants can be rescued by returning them to normal temperatures. After chilling, their rosette leaves become chlorotic, wilt, and die. In contrast, class 2 and 3 mutants eventually flower and set seed at chilling temperatures. Nevertheless, they also display a visible phenotype: The older rosette leaves of class 2 mutants become chlorotic, wilt, and die after chilling, whereas younger leaves are not affected, and the rosette leaves of class 3 mutants develop chlorotic patches. Class 1 and class 2 mutants appear to be defective in chloroplast protein accumulation at low temperatures (Schneider et al., 1995). Interestingly, the *chs1* mutants are only slightly damaged upon transfer to 5°C, suggesting that low temperature-induced lethality for these mutants may depend on high metabolic activity (Schneider et al., 1995). Although some hypotheses have been made about the affected pathways based on the observed phenotypes, the genes defective in these mutants have not yet been isolated.

To identify changes in gene expression associated with chilling damage, we have analyzed the RNA expression profiles of 8,300 genes of 12 selected chilling mutants and wild-type plants using the Arabidopsis GeneChip genome array (Zhu and Wang, 2000). These plants were grown at normal temperatures and then subjected to chilling temperatures, and the transcriptome profiles were used as a multidimensional phenotype for each mutant. The objectives were (a) to identify putative genes important for plant acclimation to moderate low temperatures by comparing the global transcript expression pattern of wild-type and chilling mutant plants with and without chilling treatment, (b) to further understand the defects in different types of chilling mutants by analyzing their molecular phenotypes, and (c) to compare the responses of Arabidopsis plants to 13°C and 4°C.

RESULTS

Changes in mRNA Accumulation in Response to Chilling in Arabidopsis

To identify molecular responses of Arabidopsis during acclimation to chilling, we performed GeneChip microarray experiments monitoring transcriptional responses of mature Arabidopsis plants upon exposure to 13°C for 2 d. Technical and biological variations (outliers that greater than 2-fold and higher than 25 average difference signal intensity between replicates) were estimated before the analysis of chilling transcriptome. The average technical variation measured by false positive rate of duplicate arrays is approximately 0.2% (Zhu et al., 2001), whereas the biological variation contributed by the growth conditions, measured by false positive rate of duplicated biological samples is 0.4% ± 0.2% (n = 10). The high correlation coefficient of 0.99 ± 0.01 was achieved by de-emphasizing the experimental and biological variations contributed by the experimental error and individual plants. In this study, replicated samples each consisting of 10 individual plants were pooled using a strategy described by Kreps et al. (2002). A total of 1,457 genes showed a 2-fold or more difference in mRNA accumulation relative to their untreated levels after chilling. Among the genes with altered expression, 532 were up-regulated, and 925 genes were down-regulated.

The 1,457 chilling-responsive genes were functionally classified using the Munich Information Center for Protein Sequences (MIPS) scheme (Mewes et al., 1999). Results from this classification including GeneChip probe set numbers, Arabidopsis Gene Index numbers, ratios, and annotations are shown in Supplemental Table 1 (supplemental material can be viewed at http://www.plantphysiol.org). Because specific differences could be masked by the fact that a given functional category was present at a low number within all genes on the microarray, we normalized the classification frequency within the 1,457 genes to the classification frequency for the entire microarray (see "Materials and Methods"). Using this approach, it was possible to identify subtle and meaningful shifts in functional classification. The normalized lists based on the MIPS classification indicated that genes under the protein synthesis and energy categories were significantly over-represented in the 532 genes that are 2-fold or greater up-regulated by chilling, as shown in Figure 1. The large number of genes in this list includes genes distributed across many functional categories, suggesting that major molecular changes were associated with the transition from warm to moderate low (chilling) temperatures.

The most evident result derived from the wild-type chilling treatment was the near 4-fold enrichment of genes involved in protein synthesis in the list of chilling-responsive genes versus the overall list of the Arabidopsis Genechip: 69 genes encoding products with predicted functions related to protein synthesis were up-regulated. Among these, 45 genes encode ribosomal proteins. These extensive changes in mRNA accumulation suggest that transcriptional regulation of the protein synthesis machinery is a critical component of plant acclimation to decreasing temperatures.

Chilling affects many genes encoding transcription factors. By examining 402 genes encoding stress-responsive transcription factors from different families (Chen et al., 2002), we have found that 90 genes

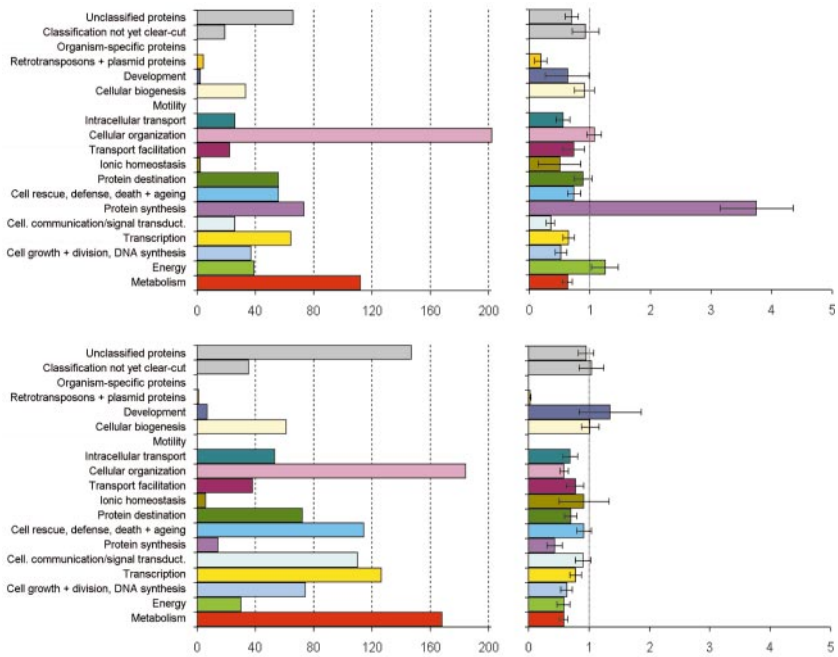


Figure 1. Functional classification of genes responding to chilling. The left panel displays the absolute number of genes (represented by probe sets) falling into each category. The right panel shows the results of normalizing the frequency for each category to the frequency of the category in the list of 8,000 genes. Top, Classification of up-regulated genes; Bottom, classification of down-regulated genes.

Table I. *Summary of set sizes and numbers in intersects*

Set sizes with changes greater than 2-fold were generated as follows. Q, Mutant untreated versus wild-type untreated; R, mutant treated versus mutant untreated; MU, treated: untreated in mutant less those in common with treated: untreated in wild type; RR, ratio of mutant treated over untreated versus ratio of wild-type treated over untreated; and TR, mutant treated versus wild-type treated. A brighter yellow color in "N in Sets" indicates higher numbers in a given set, whereas darker shading in "N in Intersection" indicates higher numbers in common between sets.

	N in Sets					*N* in Intersections					
DOWN	Q	R	MU	RR	TR	Q ∩ MU	Q ∩ RR	Q ∩ TR	MU ∩ RR	MU ∩ TR	RR ∩ TR
chs 1-1	349	1459	1104	1630	1470	14	12	58	1023	959	1348
chs 1-2	344	1610	1181	1709	1569	18	32	92	1068	1000	1407
chs 2-1	296	1227	890	1358	1278	9	16	76	772	722	1109
chs 2-2	307	1313	944	1467	1377	9	12	56	840	802	1222
chs 3	125	1025	620	1121	1079	5	12	40	497	492	910
chs 4-1	331	372	142	500	396	0	0	52	54	24	250
chs 4-2	262	584	252	671	720	3	4	83	138	133	484
chs 4-3	282	659	206	442	227	1	4	18	94	57	175
chs 5	104	692	171	367	248	0	3	13	61	45	181
chs 6-1	373	714	298	410	89	0	1	21	156	12	46
chs 6-2	432	812	376	508	199	0	1	51	200	16	96
chs 6-3	430	767	300	376	127	0	0	24	165	15	71
UP	Q	R	MU	RR	TR	Q ∩ MU	Q ∩ RR	Q ∩ TR	MU ∩ RR	MU ∩ TR	RR ∩ TR
chs 1-1	308	1231	1146	1725	1519	31	39	91	1045	1005	1381
chs 1-2	387	1093	1011	1552	1433	59	72	151	901	878	1243
chs 2-1	343	1087	990	1632	1557	62	87	174	853	816	1338
chs 2-2	259	1193	1096	1713	1524	38	54	109	991	928	1381
chs 3	190	828	719	1357	1281	76	90	127	617	616	1108
chs 4-1	311	314	175	953	790	24	66	191	118	100	545
chs 4-2	350	515	342	1090	1075	78	153	276	256	253	804
chs 4-3	82	376	195	658	318	1	1	10	114	74	248
chs 5	63	377	161	539	278	1	0	12	60	39	233
chs 6-1	488	478	264	741	527	2	9	191	132	37	243
chs 6-2	563	481	268	740	619	5	20	260	162	65	281
chs 6-3	450	598	285	736	464	1	9	192	148	44	206

were differentially expressed in chilled plants relative to the control. Seventy-eight of these were down-regulated, whereas 12 were up-regulated (Supplemental Table 1). Their potential roles in primary responses to chilling will need to be tested further.

Molecular Phenotyping of Chilling-Sensitive Mutants

To characterize the regulation of molecular processes necessary for optimal growth of Arabidopsis at moderate low temperatures, we investigated the molecular alterations in response to chilling displayed by the *chs* class of chilling-sensitive mutants (Schneider et al., 1994). We first identified chilling-regulated genes in these mutants, used their expression patterns as molecular phenotypes, and then combined groups of mutants with similar molecular phenotypes to identify those chilling-responsive genes that are essential for acclimation to chilling.

The feasibility of using mutant gene expression pattern as a consistent phenotype for mutant analysis was studied by analyzing the correlation between transcript alterations before the appearance of the visible phenotypes and the severity of visible phenotypes for the 12 chilling-sensitive mutants. This allowed us to test whether the similar phenotypes of mutants in different classes of chilling mutants were indicative of their relationship at the molecular level by the following steps. First, we generated a number of gene lists based on relative gene expression levels in the mutants compared with the wild type or the untreated mutants as a control reference (see "Materials and Methods"). Second, we compared the number of genes with altered expression levels in different mutants. The number of genes identified in each mutant and the number of genes common to different mutants are shown in Table I.

The number of genes in the lists consisting of ratios that compare gene expression in the chilled mutants and the chilled wild type, namely the treated ratio (TR; mutant treated:wild-type treated) and the ratio of ratios (RR; mutant treated:mutant untreated/wild-type treated:wild-type untreated) lists, correlated well with the severity of the visible phenotype of the mutants after chilling treatment. Accordingly, chilling-lethal class 1 mutants had larger numbers of altered genes than class 2 and 3 mutants. Moreover, the number of genes and the content of the lists for

TR and RR ratios for each mutant were very similar (Table I), indicating that these two ratios are equally suitable for determining mRNA accumulation changes in the mutants with respect to the wild type after chilling treatment.

A large number of genes were found in the mutant-unique ratio (MU; mutant treated:mutant untreated-wild-type treated:wild-type untreated) lists (Table I), which included genes with altered expression in the mutants due to chilling exposure that were not regulated by chilling in the wild type. Additionally, the intersection of the MU lists with the quiescent ratios (QR; mutant untreated:wild-type untreated) lists was very small, and the overlap of the MU list with the TR and RR lists was large (Table I). These results combined indicate that the expression of a very large number of genes (more than 1,000 for several class 1 mutants), unaffected by chilling in normal plants, was affected by chilling in the chilling-sensitive mutants. It is likely that the function of the mutated genes is necessary to prevent a massive negative effect of chilling on transcriptional regulation, different from chilling exposure effects in the wild type. This effect, combined with the alteration in components regulated by chilling can be ultimately responsible for the chilling-lethality of the mutants.

To evaluate the relationship of the transcriptome profiles among mutants within and across their visible phenotypic classes, we clustered the transcriptome profiles of 826 probe sets, representing 773 genes, that showed changes in mRNA accumulation in the wild type upon chilling and displayed altered responses (quantified by the TR) differing more than 2-fold from the wild type in at least one of the mutants (marked in Supplemental Table 1). The results of the cluster analyses of the gene expression ratio profiles of the mutants are shown in Figure 2. Similar to the results obtained from the gene lists described above, mutants belonging to class 1, the class with a lethal phenotype, displayed profiles with most genes

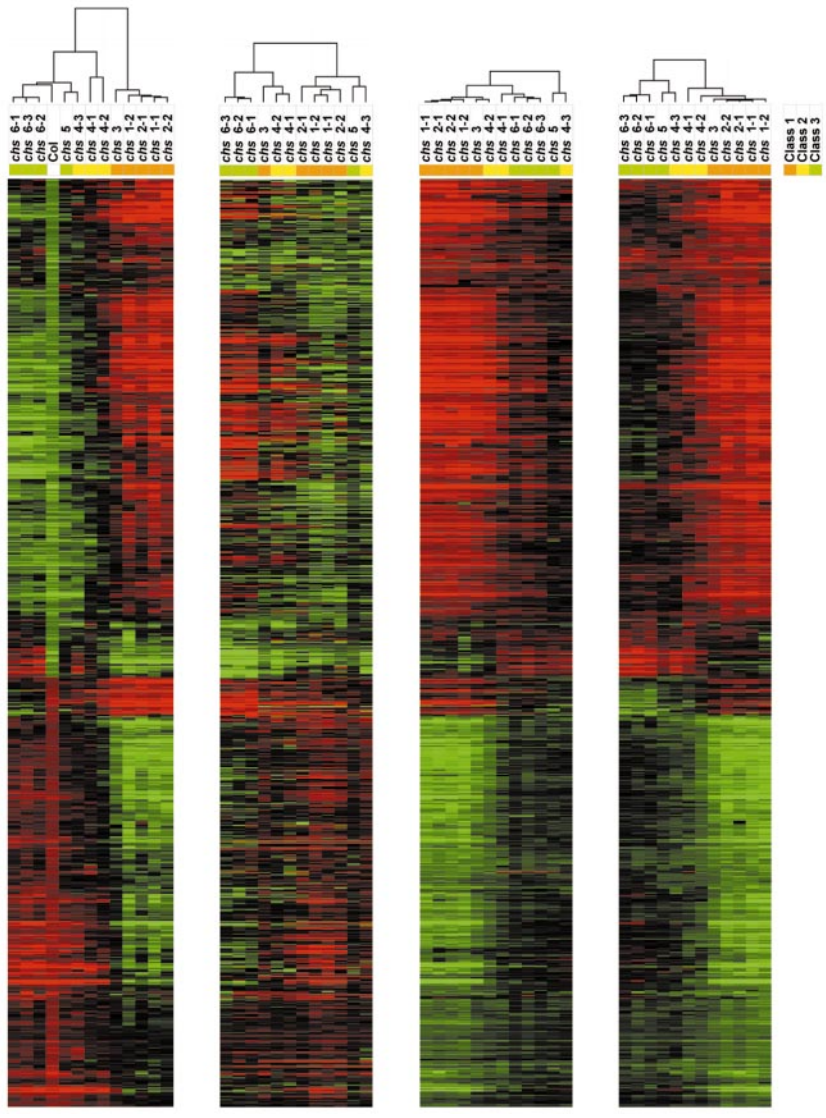


Figure 2. Cluster analysis of gene expression of chilling-responsive genes in wild-type and mutant plants. Clusters were based on the various ratios calculated as described in "Materials and Methods." Red represents an increase in relative expression, whereas green represents a decrease. The order of the genes in the three right panels corresponds to the gene cluster order in the left panel. Panels from left: R, Mutant treated versus mutant untreated; QR, mutant untreated level versus wild-type untreated level; TR, mutant treated versus wild-type treated; RR, mutant treated:untreated/wild-type treated:untreated.

having dramatically altered gene expression levels in comparison with the nonlethal chilling-sensitive phenotype classes 2 and 3. Moreover, profiles for mutants in the same class were very similar, particularly for clusters for the ratios comparing chilled samples to untreated samples (standard ratio [R]) and chilled mutants to chilled wild type (TR and RR). For R, within class 1, *chs 3* clustered separately from *chs 1-1*, *chs 1-2*, *chs 2-1*, and *chs 2-2*. *chs 1-1* and *chs 1-2* clustered together, as did *chs 2-1* and *chs 2-2*, reflecting their allelism. This supports the idea that the products of these genes may perform related biological functions. The less severe phenotype classes also showed good clustering, but were less similar to one another within each class. As expected, for class 3 mutants, *chs 6-1*, *chs 6-2*, and *chs 6-3* clustered more closely with each other than with *chs 5*. *chs 5* was closer in cluster space to the class 2 mutant *chs 4-2*. These results indicate that cluster analyses identify and group together different alleles for each *chs* loci. More importantly, these results also show that mutants affected in different genes cluster together according to a previous organization based on their visible phenotypes, with the possible exception of *chs 5*.

To quantify the similarity between mutants at the molecular level we compared the number of genes in common between lists of genes that were 2-fold or more up- or down-regulated in all mutants after chilling (TR lists). To assess the commonalities between lists of genes, we calculated the intersection of the lists of genes for all pair wise comparisons, for a total of 66 comparisons. The results of this comparison can be visualized as a pseudo-Venn diagram, as shown in Figure 3. The size of the sphere reflects the number of members in the set. Higher commonality is indicated by a thicker line connecting the spheres, as well as by a closer proximity of the spheres. The similarity ratio used for comparison was twice the number of differentially regulated genes in common to both sets divided by the sum of the number of members in both sets. Results from this analysis indicate that mutants in class 1 are a very closely related group. The 10 comparisons between the five members of this class were the top 10 highest similarity ratios among all pair wise comparisons, with similarity values for the up-regulated lists ranging from 95% to 98% (data not shown). This result suggests that all three genes defective in class 1 mutants are altering related mechanisms associated with acclimation to chilling. This analysis also indicates that in most cases, the number of genes affected in common is higher within each mutant class than across classes.

Chilling-Responsive Genes with Altered Expression in Chilling-Lethal Mutants

To identify candidates for genes whose regulation may be necessary for acclimation to the transition from warm to low temperatures in Arabidopsis, we analyzed the transcriptional regulation of the 1,457 chilling-regulated genes in the wild type and in the 12 chilling-sensitive mutants. Among them, 773 genes displayed altered responses differing more than 2-fold from the wild type in at least one of the mutants and were selected for gene cluster analyses. With respect to the clustering of the 773 genes, it was observed that two general classes of genes could be identified using either the R, which indicates the extent of the chilling response in the mutant, or the TR, which indicates differences of mRNA accumulation between the chilling-treated wild type and the

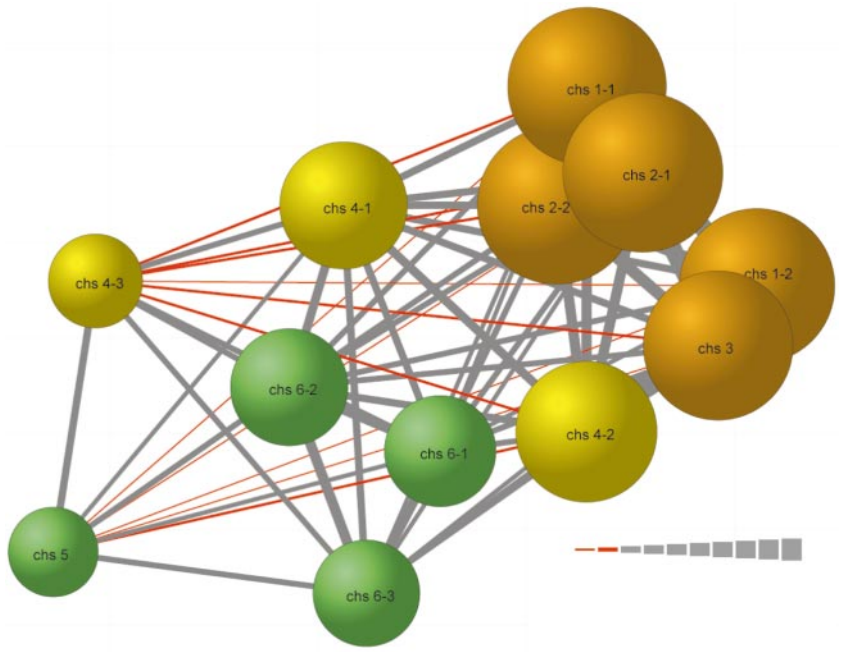
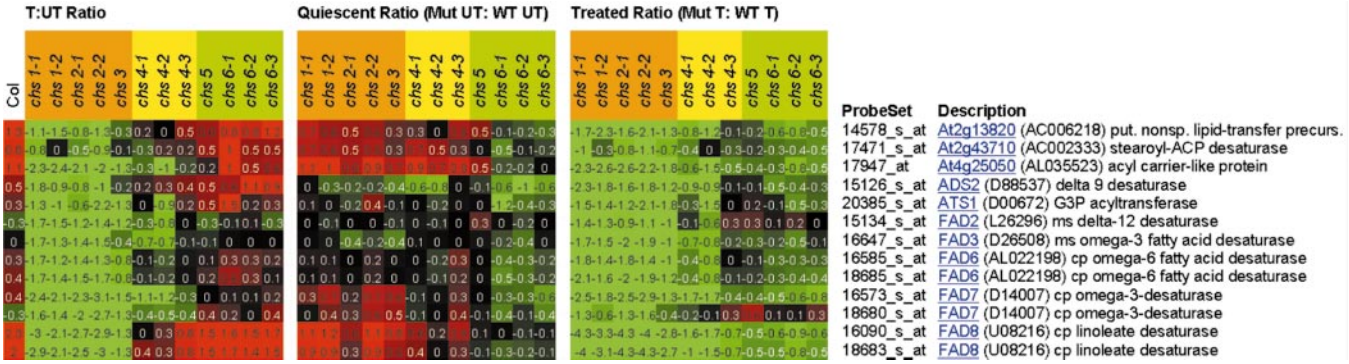


Figure 3. Genes altered by chilling in common among mutants. Commonalities analysis for up-regulated genes based on pair wise Venn-comparisons among mutants using the TR. The volume of the spheres indicates the number of genes affected using a 2-fold or higher cut-off. The thickness of the lines connecting the spheres denotes the level of commonality, score ranged from 0.1–1.0, between mutants. A red coloring for the line denotes lists with a commonality of 0.2 or less.

Table II. *Lipid metabolism genes related to low-temperature tolerance:GeneChip probe sets, annotation and relative expression levels as log_2-transformed treated:untreated ratio, QR, and TR*



chilling-treated mutant. The first class included the majority of the genes, and corresponded to those genes either up- or down-regulated in the wild type that showed a reduced response in the chilling-sensitive class 1 mutants. An inspection of the TRs indicated that after chilling, the mRNA levels for most of these genes were altered to different degrees in all three classes of mutants with respect to the levels in the wild type. However, the most extreme (i.e. smallest or largest) ratios were observed for class 1 mutants, a fact consistent with the lethality observed for these mutants after exposure to chilling. A second set of genes can be observed clustered around the line dividing genes up- and down-regulated in the wild type: These showed altered expression in class 2 and 3 mutants but little change in class 1 mutants. A good representative of this set is a drought-inducible Cys proteinase RD21A homolog (At4g11320) that showed more than 2-fold down-regulation in the wild type and four of five class 1 mutants but appeared up-regulated by more than 2-fold in all class 2 and 3 mutants. The existence of this set of genes suggests that the proteins encoded by the loci mutated in class 2 and 3 mutants are not just downstream targets of the genes mutated in class 1 mutants and supports the idea that lesions in different responses can cause sensitivity to chilling.

Although we used a chilling treatment of wild-type plants to obtain an overall analysis of the functional classes or pathways with many components responding to chilling, we established a very stringent process to select specific genes whose response to chilling was significantly affected in class 1 mutants and therefore whose expression may be critical for acclimation to grow at suboptimal temperatures. In addition, this list could also include genes related to secondary effects on metabolism that could be derived from molecular alterations on genes or gene products susceptible to low temperatures. To minimize the number of false positives related to data variation due to the low temperature treatment, we only considered genes as being related to chilling tolerance if they passed two criteria: being induced more than 2-fold by chilling in the wild type and displaying a TR of at least 2-fold in the opposite direction in all *chs1-1*, *chs1-2*, *chs2-1*, *chs2-2*, and *chs3* class 1 mutants. This approach was possible because, as shown above, all class 1 mutants displayed highly similar profiles with respect to chilling-regulated genes. From this selection 233 up-regulated genes and 401 down-regulated genes were identified. Functional classification was performed for those genes with a predicted function (Supplemental Table 2A).

Among the putative mechanisms that may be directly involved in the transition to low temperature, many of the genes in the protein biosynthesis group were negatively affected in all class 1 mutants (Supplemental Table 2A). Among at least 18 ribosomal proteins affected in class 1 mutants, the strongest induction by chilling in the wild type was shown by a 60S ribosomal protein L14 (At2g20450), with more than 5.2-fold induction. At least two putative chloroplastic ribosomal proteins (At2g24090 and At3g44890) were also present in this group. There was also a dramatic increase in transcript abundance for genes involved in other aspects of protein biosynthesis such as two genes encoding tRNA synthetases and 10 genes encoding RNA helicases and translation initiation factors. In addition, protein degradation may also be affected as indicated by the down-regulation of genes encoding a ubiquitin (At4g05320) and a ubiquitin-like protein (At4g12570).

Another possible direct response to a reduction in temperature that can be affected by mutations provoking chilling-sensitivity is the change in lipid content due to altered fatty acid desaturation. Such metabolic processes are potentially responsible for structural changes in cellular membranes and have

been shown to be determinant for plant tolerance to low temperatures. We found three genes related to lipid metabolism up-regulated by chilling with a suppressed response in the class 1 mutants: the *FAD8* gene encoding a chloroplast linoleate desaturase, an acyl carrier-like protein gene (At4g25050), and a nonspecific lipid-transfer protein gene (At2g13820). Because of the previous knowledge about the role of genes related to lipid desaturation during low temperature tolerance, we have also analyzed in detail the expression levels of several other genes that might be involved in this process (Table II). These results indicate a reduced expression after chilling of the *FAD2*, *FAD3*, *FAD6*, *FAD7*, *FAD8*, and *ATS1* genes in most of the class 1 mutants compared with the treated wild type and the untreated mutant (TR and R ratios). These results indicate a possible defect in the metabolism of 16:3 and 18:3 fatty acids. Interestingly, the study of a triple mutant *fad3 fad7 fad8* with defects in ω3-desaturases indicated that trienoic acids are required to maintain chloroplast structure and normal photosynthesis at low temperatures (Routaboul et al., 2000). A defect could also be observed in the chilled mutants for the accumulation of the transcript encoding a stearoyl-ACP desaturase, catalyzing the first committed step for the biosynthesis of C18-unsaturated fatty acids, and *ADS2*, encoding a cold-induced Δ9-desaturase (Fukuchi-Mizutani et al., 1998). The class 2 mutants are only defective in the expression of the *FAD7* ω3-desaturase, and in class 3 mutants, no significant decrease in the expression of desaturase genes was detected. These data do not support the idea that changes in fatty acid desaturation mediate chilling responses and determine mutant phenotypes related to chilling sensitivity in classes 2 and 3 *chs* mutants. Nevertheless, such changes can be a critical factor for the lethality of class 1 mutations.

On the basis of previous studies, it is likely that molecular components implicated in the detoxification of AOS are likely to be among processes responsible for the inability of the chilling-lethal mutants to acclimate to reductions in temperature in the light (Iba, 2002). The amount of AOS in plants increases when they are exposed to low temperatures, high-light intensity, and other stresses. In our analysis, we have identified misregulation of chilling-responsive genes encoding detoxifying activities, such as peroxidase and iron superoxide dismutase (Supplemental Table 2A), in class 1 mutants in response to chilling that could lead to chloroplastic oxidative damage and the subsequent chlorotic phenotype. Supporting this possibility, it has been shown that iron superoxide dismutase can protect a cyanobacterium species against chilling damage (Thomas et al., 1999).

In addition to the numerous genes with significantly altered expression in the chilling mutants with putative functions related to chloroplast development and normal function (Supplemental Table 2A), we also investigated global chloroplast-related defects in the chilling-sensitive mutants due to the critical role of the optimization of photosynthesis in response to low temperatures in the light. We monitored the expression of all genes in the GeneChip that are nuclear-encoded but whose gene products are chloroplast targeted. For such proteins, 1,102 genes were identified based on their annotation and on the predictions in TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) as available from MIPS. Of these, 249 showed 2-fold or more change in the wild type after chilling. The R of expression for each gene in the wild type and in the mutants is shown in Figure 4. More than two-thirds of these genes responded in a direction different in all of the class 1 mutants compared with the direction of the response in wild type, suggesting a transcriptional defect responsible, at least in part, for the deficiency in the accumulation of chloroplastic proteins reported for the *chs1* mutant (Schneider et al., 1995). In most cases, the response of nuclear-

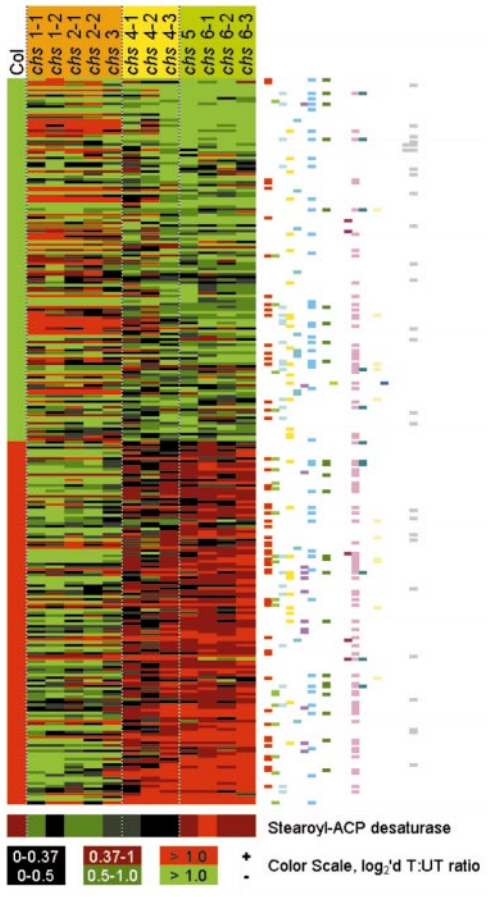


Figure 4. Expression of nuclear-encoded chloroplast-targeted genes. Two hundred and forty-nine nuclear-encoded chloroplast-targeted genes showing 2-fold or more change in wild type, and the corresponding expression levels in mutants. The right column indicate the color codes for functional categories based on the MIPS classification shown in Figure 1.

encoded genes whose products are targeted to the plastids was abolished in class 2 mutants, whereas in class 3 mutants, their response was very similar to that of the wild type.

Many of the molecular effects of the chilling-sensitive phenotype shown in Supplemental Table 2A, under the broad spectrum of carbohydrate metabolism are also likely related to changes in photosynthesis and primary metabolism. It is noteworthy that many genes involved in carbon fixation, glucolysis/gluconeogenesis, and starch and Suc metabolism are affected by chilling. For example, phospho*enol*pyruvate carboxylase, glyceraldehyde-3-phosphate dehydrogenase, sedoheptulose-1,7-bisphosphatase, a putative Fru bisphosphate aldolase (At2g21330), Glc-6-phosphate isomerase, hexokinase *ATHXK2*, a Suc-phosphate synthase-like protein (At4g10120), a glucosidase (At4g27820), and a putative isoamilase (At1g03310) were induced by chilling and were all down-regulated in class 1 mutants. An interesting response to chilling was also observed for genes involved in trehalose biosynthesis that were up-regulated, such as a trehalose-6-P phosphatase (At5g51460) and a trehalose-6-P synthase-like protein (At4g17770), and a trehalase isolog (At4g24040) that was down-regulated. This is important because cold-induced trehalose synthesis in microorganisms is essential for viability at low temperatures (Kandror et al., 2002).

Among those gene expression responses that may represent a general response to stress, the expression of genes encoding enzymes for flavonoid biosynthesis was clearly affected by chilling, with 10 genes for enzymes in this pathway up-regulated in the wild type being down-regulated in the mutants (Supplemental Table 2A). These genes displayed some of the highest levels of induction by chilling observed in this study, with seven genes exhibiting more than 10-fold increase in expression in the wild type. This coregulation is probably mediated by the myb-related transcriptional regulator encoded by the *PAP1* gene, up-regulated by chilling, that has been shown to regulate phenylpropanoid biosynthesis (Borevitz et al., 2000). Among the genes regulated by chilling and suppressed in the class 1 mutants whose expression is know to be affected by other stress responses, we found genes such as *ERD5*, a dehydration-regulated Pro oxidase (Kiyosue et al., 1996), supporting a possible contribution of Pro homeostasis to chilling acclimation. In addition, we observed altered expression of multiple putative dehydration-responsive genes including the *ERD1* and *RD19A* genes encoding proteases. With respect to biotic stress, we have observed that class 1 mutants were affected in the down-regulation by chilling of 36 defense-related genes. This list includes a gp91phox homolog (At5g47910) and pathogen-induced genes such as those encoding several glucanases and chitinases (Supplemental Table 2AB). These data are consistent with the idea that some pathogen-defense responses, perhaps including the oxidative burst, could be reduced after exposure to chilling.

Among the genes with the highest level of induction by chilling in the wild type and altered expression pattern in class 1 mutants are those with putative roles related to cell extension and cell wall metabolism (Supplemental Table 2A). The differential effect of chilling on the wild type and class 1 mutants for these genes can be interpreted as a secondary effect derived from overall reduction in metabolism and is consistent with the cessation of growth observed for these mutants at restrictive temperatures. An example is a xyloglucan endotransglycosylase (At3g44990) gene induced more than 70-fold by chilling. Other related up-regulated genes in the list encode three expansins (At2g20750, At2g03090, and At3g29030), two cellulose-synthase isologs (At4g23990, and At4g24010), three putative pectinesterases (At5g26670, At2g46930, and At4g22010), and two polygalacturonases (At1g60590 and At1g70370). Interestingly, it is also possible that growth responses during chilling under light could be coordinated by hormones. The expression of several genes encoding enzymes related to hormonal biosynthetic or signaling pathways was found to be regulated by chilling and altered in class1 mutants (Supplemental Table 2A). Flux through the GA biosynthetic pathway is likely to be increased by chilling because the expression of the *GA4* gene (At1g15550), a GA 3β-hydroxylase, was up-regulated, and that of a GA2-oxidase (At1g30040) was down-regulated. The mRNA levels for these two genes have been shown to change in response to light and GA levels. Thus, they encode enzymes that are thought to catalyze key steps regulating GA accumulation (Hedden and Phillips, 2000).

With respect to ethylene and cytokinin biosynthesis, a putative 1-aminocyclopropane-1-carboxylate oxidase (At1g03400) and a cytokinin oxidase (responsible for cytokinin degradation) were also down-regulated by chilling. In addition, the expression of *ERS*, encoding a response sensor in the ethylene signal transduction pathway (Hua et al., 1995), and two response sensors very similar to those involved in cytokining signaling (Hwang and Sheen, 2001) were down-regulated. It is also possible that these genes are related to a putative temperature sensor. In cyanobacteria, the identification of two putative sensor His kinases and a response regulator has recently demonstrated the participation of two component systems in the transcriptional control of low-temperature-regulated gene expression (Suzuki et al., 2000). Among other signaling-related genes down-regulated by chilling, we found multiple GTP-binding and calcium-related proteins, including calcium-transporters, calmodulin-like proteins, and calcium-dependent protein kinases (Supplemental Table 2A). This is interesting because cold-shock results in a rapid rise in the cytosolic-free calcium

concentration in plants that are both cold insensitive (Arabidopsis) and cold sensitive (tobacco), and a change in the calcium "signature" after acclimation has been observed, suggesting a memory-type mechanism for exposure to cold (Knight et al., 1996).

A second approach to identify genes that may be required for chilling tolerance of Arabidopsis plants was to select those genes affected in all of the class 1 mutants among those genes that are not transcriptionally regulated by chilling. The correct expression of these genes may be necessary to provide the physiological background necessary to acclimate to chilling. To address this point, an additional list was generated based on the QR of the class 1 mutants. Seventy-nine genes belonging to diverse functional classes were affected in all class 1 mutants grown at 22°C compared with the wild type (Supplemental Table 2B). Among them, only 18 genes were not regulated by chilling in the wild type.

In the long run, perhaps the most valuable information will reside in the large remaining set of genes affected in all of the class 1 mutants shown in Supplemental Tables 2A and 2B for which no clear function during chilling can be predicted at the present time. Further experiments will be necessary to fully evaluate this resource.

Changes in mRNA Levels of Cold-Responsive Genes during Chilling Responses

The expression of known cold-responsive genes was monitored closely to examine their role in the chilling response. More specifically, the response of cold-regulated genes and their possible involvement in the chilling-sensitivity of the different classes of mutants was evaluated. We compared our results with data sets obtained from two recently published studies (Fowler and Thomashow, 2002; Kreps et al., 2002). These two microarray studies allowed the identification of genes that respond consistently to cold treatments across different experimental conditions and developmental stages after short or long exposures to 4°C. We identified 137 cold-responsive genes common to both experiments and analyzed the transcriptome profiles of those detected in our chilling response experiments (Supplemental Table 3). We found 35 genes that were similarly responsive to cold and chilling. Of the 17 genes up-regulated by chilling in the wild type, eight were down-regulated more than 2-fold in all of the class 1 mutants with respect to the treated wild type. This list includes long-term cold-responsive genes: the *CHS*, *F3H*, and *FS* genes of the flavonoid biosynthetic pathway, and the early light-inducible protein (*ELIP1*) gene; as well as transiently expressed genes: an amidophosphoribosyl transferase (At4g34740), a putative β-glucosidase (At4g27820), a kinase (At2g18890), and a cytochrome p450 (At4g37320). Of 18 genes down-regulated in the wild type, six were suppressed in all class 1 mutants, including a putative thioredoxin (At4g26160), a kinase (At1g11050), a NAC-like transcription factor (At1g69490), and a peroxidase, *PRXR2* (At4g37520). However, the overall correlation between expression levels of cold-responsive genes in response to chilling and cold was poor. For example, the correlation coefficient between the relative expression data of 27 h of cold and 48 h of chilling was −0.0106, with 80 genes reacting in opposite ways. This was in contrast to a better correlation between the untreated wild-type controls in the chilling and cold experiments ($r^2 = 0.693$).

The fact that many genes reacted to the chilling response, but in the opposite way to the mature plants after 1-d cold treatment, suggested a possible involvement of those genes in the chilling response. It also prompted us to investigate their profiles in the chilling-sensitive mutants in more detail. A closer look at the mRNA accumulation of all up-regulated cold-responsive genes (Fig. 5) indicates that there is a large cluster of genes, including many of those regulated by the overexpression of the CBF factors (Fowler and Thomashow, 2002), that appear down-regulated or unaffected in the wild type after chilling

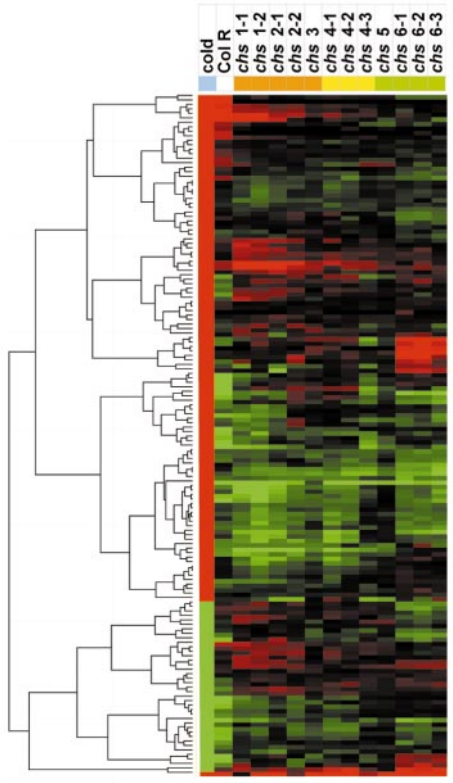


Figure 5. Cluster analysis of cold-responsive genes comparing the expression levels in the chilling mutants grown at 22°C and the wild type grown at 22°C. Column Col R represents $\log_2$-transformed wild-type treated:wild-type untreated ratio, and 12 rightmost columns correspond to the quiescent levels of these genes ($\log_2$-transformed mutant untreated: wild-type untreated ratio) in the mutants.

for 2 d. It appears that their expression is also reduced to different degrees with respect to wild-type levels in most of the untreated chilling mutants. Of those, long term cold-responsive genes *COR28*, *COR47*, *COR78*, and a putative sugar transporter (At1g08890) as well as the transiently expressed *GST30/ERD9* and unknown protein (At2g41190) displayed QR, indicating a 2-fold reduction of mRNA accumulation in all class 1 mutants. Moreover, 15 cold-responsive up-regulated genes were significantly down-regulated in all chilled class 1 mutants with respect to the chilled wild type. These genes encode *CBF3*, *COR15b*, a putative galactinol synthase, a Δ9-desaturase (from *ADS2*), *GST30/ERD9*, the response regulator *ARR7*, the AP2 domain putative transcription factor *RAP2.7*, a UDP rhamnose-anthocyanidin-3-glucoside, a putative water channel, a Suc synthase, a putative sugar transporter (At1g08890), and several other genes of unknown function. This list includes long-term and transiently expressed cold-responsive genes as well as members of the CBF regulon (Fowler and Thomashow, 2002).

DISCUSSION

Recently, the combination of genetic and transcription profiling analyses of mutants has become a powerful tool for dissecting the transcriptional component of the molecular networks underlying cellular functions in eukaryotic organisms (DeRisi et al., 1997). Using yeast as a model system, several approaches have been developed including the comparison of mutant and wild-type profiles to identify genes whose expression is affected by the mutation (Holstege et al., 1998) and the identification of groups of genes with similar patterns of expression across different mutants (Hughes et al., 2000). In Arabidopsis, similar strategies have been applied to identify downstream molecular components of a gene encoding a MAP kinase (Petersen et al., 2000) that is required for disease resistance, and to analyze the gene expression regulation by stress-responsive transcription factors (Chen et al., 2002). Moreover, transcription profiles of functionally related genes have been used in genetic analysis to establish relationships between molecular components of signal transduction pathways (Gil et al., 2001; Yin et al., 2002). In this study, we report the use of a combination of bioinformatic tools to dissect and compare the molecular phenotypes of the wild type and a collection of 12 chilling-sensitive mutants of Arabidopsis upon chilling that served to identify chilling-responsive genes with an altered response in closely related chilling-lethal mutants. Subsequently, we used microarray data from previously reports results to compare transcriptome profile changes upon chilling and cold treatments.

The most significant result from our functional analysis of chilling-responsive genes in wild-type plants is that the up-regulation of a large number of genes involved in protein synthesis is a major effect during acclimation to chilling. In all of the chilling mutants analyzed, these genes, including several chloroplastic ribosomal genes, are either not induced or are down-regulated, indicating a possible global defect in the regulation of protein biosynthesis in response to chilling. In cyanobacteria, many genes involving protein synthesis, proper folding, and processing are related to low temperature acclimation. These include those encoding RNA-binding proteins (which presumably act analogously to members of the bacterial Csp family of RNA chaperones and could be necessary for translation under cold stress), cold-induced RNA helicases (that could remove secondary structures of RNAs induced by cold), the cold-inducible family of CIp proteins (that appears to be involved in the proper folding and processing of proteins [Los and Murata, 1999]), and fatty acid desaturases. Similarly, genes involving suppression of protein synthesis and regulation of lipid composition of cellular membranes are also induced by cold in mammalian cells (Fujita, 1999). Although our results indicated that the levels of mRNA of several known genes involved in fatty acid desaturation were not dramatically altered in the chilled wild-type plants, the expression of several of these genes were affected in class 1 mutants. Taken together, our results suggest that some basic low-temperature responses may be conserved among all eukaryotic organisms.

Although the mutant loci for the 12 chilling sensitive have not been isolated, our transcriptome-profiling results provide valuable molecular phenotypic information that supports and extends the visual examination of plants. In fact, the classification of the mutants after cluster analysis and/or Venn selection based on their transcriptome profiles was highly consistent with their previous classification based on their visible phenotype, despite the lack of visible phenotype at the time when the samples were collected. Gene expression profiles have been used to classify different cancers (Alizadeh et al., 2000; Sorlie et al., 2001) and to predict the clinical outcome of the disease in breast cancer (van't Veer et al., 2002). In our case, we show that molecular phenotypes obtained from transcriptional profiling predict later visible phenotypes and can be used to classify related mutants on the basis of their molecular defects. The fact that visible differences between the wild type and the mutants are lacking after 2 d of chilling indicates that transcription profiling was a more sensitive method than visible phenotyping. Our results indicate that expression profiling is a useful tool for distinguishing mutants with similar physical phenotypes.

With respect to genes with altered expression in the *chs* mutants, our functional analyses suggest a number of candidates for chilling-responsive genes whose correct expression can be important for the transition to low temperatures. In addition, we discovered that

large numbers of chilling-altered genes in the mutants are not regulated by chilling in the wild type under the conditions tested. Transcriptome profiles of the chilling-lethal class 1 mutants indicate, in addition to the large defect in the transcriptional regulation of protein synthesis genes, a widespread transcriptional defect in chilling responses of genes whose gene products are chloroplast targeted. A defect in chloroplast protein accumulation upon chilling was previously reported for the *chs1-1* mutant, and it was hypothesized that it was caused by a direct effect on protein accumulation (Schneider et al., 1995). The altered pattern of expression of nuclear-encoded chloroplast genes in the mutants suggests a disruption in the chloroplast-nucleus cross-talk or an effect on temperature-sensitive components of the transcription machinery. This could be related to chilling-induced disruption of chloroplast function in the chilling-sensitive mutants. Most notable are the alteration of enzymatic activities necessary for protection against oxidative stress and the aberrant regulation of components of the carbon-fixation machinery and other carbohydrate metabolism pathways that may result in changes in photosynthetic rates and photoxidative injuries (Stitt and Hurry, 2002). This interpretation is supported by the chilling-induced degradation of chlorophyll and chloroplasts in the *chs 1-1* mutant (Patterson et al., 1993).

Although our primary goal is to uncover mechanisms of acclimation to moderate low temperatures in plants, the combination of our data and other studies offers an opportunity to compare transcriptome differences in downstream responses after chilling and colder treatments. This analysis suggests that qualitative differences exist between these two stresses. In the wild type of a similar age, the correlation coefficient of chilling-induced genes is negative when compared with 1-d cold (4°C) treatments. However, the altered expression of some cold-responsive genes in the chilling-sensitive mutants suggests that some of these genes can be affected by chilling despite the fact that they appeared unaffected at the 2-d time point of our experiments. This effect can perhaps be explained by delayed induction kinetics, dose-response differences at different temperatures or the transient nature of their expression. Although freezing tolerance of these chilling mutants has not been tested, Schneider et al. (1995) reported that the chlorotic phenotype of *chs 1-1* is visible only under certain temperatures, e.g. at 13°C, whereas at lower temperatures, e.g. 5°C, the chlorotic phenotype does not occur, suggesting that under conditions of low metabolic activity such as 4°C, these mutants may display responses to cold similar to the wild type. Therefore, a possible explanation for the existence of common and different components during cold and chilling responses may be related to the fact that a 13°C treatment for 2 d is not likely to be sufficient to initiate cold acclimation (Lee et al., 1999) and only includes responses to acclimate plant metabolism to grow at low, above-freezing temperatures.

MATERIALS AND METHODS

Seeds of Arabidopsis wild type and chilling-sensitive mutants *chs 1-1* (cs3097, PM11), *chs 1-2* (cs6252, ST106), *chs 2-1* (cs6298, PM2), *chs 2-2* (cs6299, ST117), *chs 3* (cs8000, ST119), *chs 4-1* (cs8001, ST13), *chs 4-2* (cs8002, ST35), *chs 4-3* (cs8003, ST64), *chs 5* (cs8004, ST34), *chs 6-1* (cs8005, ST39), *chs 6-2* (cs8006, ST48), and *chs 6-3* (cs8007, ST83) of the Columbia ecotype were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio). Seeds were geminated in Metro-Mix soil (Scotts-Sierra Horticultural Products Co., Marysville, OH) in flats and were grown in controlled-environment chambers CMP4030 (Conviron, Winnipeg, Canada) at 22°C under a 12-h/12-h light/dark regime and 80% humidity. Plants received approximately 350 μmol s^{-1} m^{-2} of light from two light banks emitting 15.069 lux or 45.2 W m-2. At the 4th week, one-half the number of the mutants and wild-type plants was transferred to another growth chamber at 13°C for 2 d before samples were harvested. The condition in this growth chamber is identical with the condition described, except the temperature. Samples from 10 individual treated and control plants were harvested between 11 AM and 1 PM and were pooled. All aerial material was harvested for untreated and chilling-treated plants. Mutant plants showed no visible phenotype at this point.

For Arabidopsis GeneChip experiments, RNA samples were extracted, and subsequent cDNA synthesis, array hybridization, and overall intensity normalization for all of the arrays for the entire probe sets were performed as described by Zhu et al. (2001).

Differentially expressed genes were selected based on two criteria: expression level measured as average difference of the probe set greater than 25 and 2-fold changes as compared with the corresponding control. To identify genes whose expression was affected by chilling in the wild type, wild-type plants grown at 22°C were used as a control. To identify genes whose expression was altered in the mutants, wild-type plants with chilling treatment (for TR) or the appropriate untreated mutant plants (for simple R) were used as controls.

Predicted gene functions were assigned using the annotation based on either previous experimental studies or sequence homology to known genes of other organisms. The function of the genes in their respective lists was based primarily on their annotation and functional classification. Functional classification of the genes in a given list was based on the hierarchical MIPS functional classification scheme (Mewes et al., 1999). The current MIPS classifications for 25,450 Arabidopsis genes as of March 13, 2002, was downloaded as an XML file from http://biors.gsf.de:8111/searchtool/searchtool.cgi. A conversion table was used to convert the GeneChip IDs to Arabidopsis Gene Index numbers. With the MIPS classification, a sequence can be in multiple classification categories, because the categories are not mutually exclusive. Those IDs falling into classification categories other than unclassified and classification not yet clear-cut were removed from these two categories. For the genes in a given list, the frequency for each functional class was calculated by dividing the number of genes (as represented by probe sets) falling into each functional class by the total number of genes (as represented by probe sets) in the list belonging to any class. This value was then normalized to the frequency of the given functional class over all GeneChip IDs. The resultant value was called the class score:

$$ClassScore = (N_{class[inputset]}/N_{classified[inputset]})/(N_{class[8K]}/N_{classified[8K]})$$

A class score greater than one indicates that class is over-represented in the list in question, whereas a value of less than one indicates that class is underrepresented in the list in question, both relative to the prevalence of genes in that class in the set of all classified genes on the 8K chip. To generate a reliability measure for each class score, the input set was bootstrapped 100 times by sampling randomly (with repeats) n times from the input set, where n is the number in the input set, and then calculating the class scores for each bootstrapped set. The average score and SD were then calculated, and the SD was used to estimate the reliability of the class score.

Cluster analysis was performed according to Eisen et al. (1998). First, ratios were $\log_2$ transformed. This was followed by self-organizing map generation (Tamayo et al., 1999) and subsequent hierarchical clustering with an uncentered correlation similarity metric.

Venn selection was performed using a custom script that calculates all possible commonalities between pairs, threesomes, foursomes, etc. for up to 12 different lists. Commonalities, as calculated using twice the number in the intersection divided by the number in set 1 plus the number in set 2, were examined for lists for each mutant within a given set, as well as for mutant lists in different sets. The term "set" refers to a way of calculating a given ratio and subsequent filtering based on a 2-fold change or higher cutoff criterion: Five different sets were generated: QR (mutant untreated level versus wild type untreated level), R (treated versus untreated) ratio, MU set (treated:untreated in mutant less those in common with treated: untreated in wild type), RR (mutant treated:untreated/wild type treated: untreated), and TR (mutant treated versus wild type treated). The data for the cold-treated plants were obtained from Kreps et al. (2002) and Fowler and Thomashow (2002). Data filtering was performed as described.

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