Metabolic Pathways Involved in Cold Acclimation Identified by Integrated Analysis of Metabolites and Transcripts Regulated by DREB1A and DREB2A[1][W][OA]

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DREB1A/CBF3 and DREB2A are transcription factors that specifically interact with a cis-acting dehydration-responsive element (DRE), which is involved in cold- and dehydration-responsive gene expression in Arabidopsis (*Arabidopsis thaliana*). Overexpression of *DREB1A* improves stress tolerance to both freezing and dehydration in transgenic plants. In contrast, overexpression of an active form of DREB2A results in significant stress tolerance to dehydration but only slight tolerance to freezing in transgenic plants. The downstream gene products for DREB1A and DREB2A are reported to have similar putative functions, but downstream genes encoding enzymes for carbohydrate metabolism are very different between DREB1A and DREB2A. We demonstrate that under cold and dehydration conditions, the expression of many genes encoding starch-degrading enzymes, sucrose metabolism enzymes, and sugar alcohol synthases changes dynamically; consequently, many kinds of monosaccharides, disaccharides, trisaccharides, and sugar alcohols accumulate in Arabidopsis. We also show that *DREB1A* overexpression can cause almost the same changes in these metabolic processes and that these changes seem to improve freezing and dehydration stress tolerance in transgenic plants. In contrast, *DREB2A* overexpression did not increase the level of any of these metabolites in transgenic plants. Strong freezing stress tolerance of the transgenic plants overexpressing *DREB1A* may depend on accumulation of these metabolites.

Low temperature and dehydration are adverse environmental conditions that affect plant growth and productivity. Many genes have been described that respond to both stresses at the transcriptional level, and their gene products are thought to function in stress tolerance and response even though these stresses are quite different (Thomashow, 1999; Zhu, 2002; Shinozaki and Yamaguchi-Shinozaki, 2003; Bartels and Sunkar, 2005). These genes include key metabolic enzymes, late embryogenesis-abundant (LEA) proteins, detoxification enzymes, chaperones, protein kinases, and transcription factors (Thomashow, 1999; Zhu, 2002; Shinozaki and Yamaguchi-Shinozaki, 2003; Bartels and Sunkar, 2005). The cis-acting elements that function in stress-responsive gene expression have been analyzed to elucidate the molecular mechanisms of gene expression in response to these stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). The dehydration-responsive element (DRE), containing the core sequence A/GCCGAC, was identified as a cis-acting element that regulates gene expression in response to both dehydration and cold in Arabidopsis (*Arabidopsis thaliana*; Yamaguchi-Shinozaki and Shinozaki, 1994). A similar motif was identified as the C-repeat and low temperature-responsive element in promoter regions of low temperature-inducible genes (Baker et al., 1994; Jiang et al., 1996).

Arabidopsis cDNAs encoding the ethylene-responsive element-binding factor/APETALA2 (ERF/AP2)-type DRE-binding (DREB) proteins CBF1, DREB1A, and DREB2A have been isolated by yeast one-hybrid screening (Stockinger et al., 1997; Liu et al., 1998). They specifically bind to the DRE/C-repeat sequence and

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activate transcription of genes driven by the DRE sequence in Arabidopsis. Three DREB1/CBF proteins are encoded by genes that lie in tandem on chromosome 4 in the order *DREB1B/CBF1*, *DREB1A/CBF3*, and *DREB1C/CBF2* (Gilmour et al., 1998; Liu et al., 1998). Arabidopsis also has two DREB2 proteins, DREB2A and DREB2B (Liu et al., 1998). Although expression of all three *DREB1/CBF* genes is induced by cold but not by dehydration, both *DREB2* genes are induced by dehydration and high salinity (Liu et al., 1998). Both DREB1/CBF and DREB2 proteins bind to DRE, but DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in dehydration-responsive gene expression.

Overexpression of DREB1/CBFs driven by the cauliflower mosaic virus *35S* promoter increases stress tolerance to freezing, dehydration, and high salinity in transgenic Arabidopsis (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). More than 40 downstream targets of DREB1A/CBF3 have been identified by microarrays (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). Overexpression of the constitutively active form of DREB2A (*35S*:*DREB2A-CA*) significantly increases dehydration tolerance but only slightly increases freezing tolerance. Microarray analyses of the *35S*:*DREB2A-CA* plants revealed that DREB2A regulates the expression of many dehydration-responsive genes. However, some genes regulated by DREB1A are not regulated by DREB2A (Sakuma et al., 2006a).

Promoter analysis of DREB1A- and DREB2A-regulated genes and gel mobility shift assays revealed that DREB1A and DREB2A have different DNA-binding specificities. DREB1A has highest affinity for A/GCCGACNT, whereas DREB2A preferentially binds ACCGAC (Maruyama et al., 2004; Sakuma et al., 2006a). This affinity controls the induction of different downstream genes of DREB1A and DREB2A. Recently, Sakuma et al. (2006b) reported that DREB2A expression is also regulated by heat shock (HS) stress and that thermotolerance is significantly increased in *35S*:*DREB2A-CA* plants. HS-inducible genes such as HS proteins and HS transcription factors are also upregulated in *35S*:*DREB2A-CA* plants but not in *35S*:*DREB1A* plants. Although DREB2A-regulated genes are involved in dehydration, high salinity, and HS stress tolerance, they are not sufficient to withstand freezing stress. DREB1A and DREB2A downstream gene products mostly have similar functions, but downstream genes encoding enzymes for carbohydrate metabolism are different between DREB1A and DREB2A. Several large-scale analyses have investigated metabolites in cold-exposed and *35S*:*DREB1A* plants (Cook et al., 2004; Kaplan et al., 2004, 2007; Hannah et al., 2006). The levels of many metabolites (e.g. carbohydrates, amines, and organic acids) increase significantly in both cold-exposed and *35S*:*DREB1A* plants. However, there has been no reported effort to perform large-scale metabolite analyses in dehydration-exposed and *35S*:*DREB2A-CA* plants.

Here, we performed integrated analysis of both metabolites and transcripts in dehydration-exposed and *35S*:*DREB2A-CA* plants and compared them with those in cold-exposed and *35S*:*DREB1A* plants. We also analyzed the expression of genes involved in carbohydrate metabolism under both cold and dehydration conditions.

RESULTS

Identification of Metabolites in Various Plants

We measured metabolites using gas chromatography-time of flight-mass spectrometry (GC-TOF-MS), capillary electrophoresis-mass spectrometry (CE-MS), and liquid chromatography-ion trap-mass spectrometry (LC-IT-MS) in eight kinds of plants: untreated, cold exposed (1 and 4 d), dehydration exposed (2 and 3 d), *35S*:*DREB1A*, *35S*:*DREB2A-CA*, and control plants containing pGreen. In GC-TOF-MS and CE-MS analyses, 254 independent metabolites were identified by retention time indices and specific mass fragments (Supplemental Fig. S1). The levels of 155, 162, 37, and 28 metabolites increased significantly in cold-exposed, dehydration-exposed, *35S*:*DREB1A*, and *35S*:*DREB2A-CA* plants, respectively (Benjamini and Hochberg false discovery rate [FDR]; $P < 0.01$). The levels of 50, 85, nine, and 38 metabolites decreased significantly in cold-exposed, dehydration-exposed, *35S*:*DREB1A*, and *35S*:*DREB2A-CA* plants, respectively (FDR; $P < 0.01$; Supplemental Table S1).

Venn diagrams illustrate the identified metabolites in the plants (Fig. 1; Supplemental Fig. S2). The levels of 102 metabolites increased in both cold-exposed and dehydration-exposed plants. The levels of 17 metabolites increased in both *35S*:*DREB1A* and *35S*:*DREB2A-CA* plants. Most metabolites (89%) that increased in *35S*:*DREB1A* plants also increased in cold-exposed plants. These metabolites contained sugars such as Suc, galactinol, myoinositol, raffinose, and unknown metabolites. Likewise, most metabolites (89%) that increased in *35S*:*DREB2A-CA* plants also increased in dehydration-exposed plants. In this case, these metabolites contained organic acids such as argininosuccinate, fumarate, malic acid, and unknown metabolites. In addition, most metabolites that were almost unchanged in *35S*:*DREB1A* plants were also unchanged in *35S*:*DREB2A-CA* plants; unfortunately, these metabolites are unknown (Fig. 1).

LC-IT-MS analyses showed that accumulation of three secondary metabolites increased significantly in the cold-exposed, *35S*:*DREB1A*, and *35S*:*DREB2A-CA* plants but not in the dehydration-exposed plants (Supplemental Fig. S3). Mass fragment/retention time comparisons revealed these three secondary metabolites as kaempferol 3-[6′-(glucosyl) rhamnoside] 7-rhamnoside, kaempferol 3-glucoside 7-rhamnoside, and kaempferol 3-rhamnoside 7-rhamnoside.

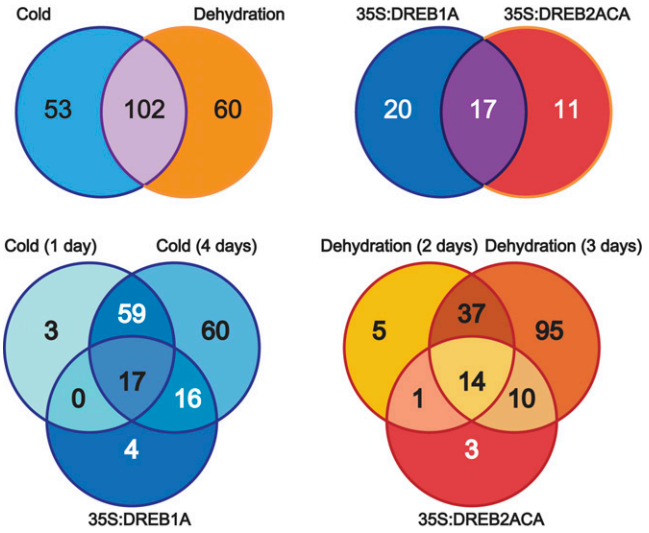

Figure 1. Venn diagrams of identified metabolites that are increased relative to the controls. The diagrams illustrate the number of identified metabolites in six kinds of plants: cold exposed (1 and 4 d), dehydration exposed (2 and 3 d), *35S:DREB1A*, and *35S:DREB2A-CA*.

Seventeen Metabolites Were Unique to Both Cold-Exposed and *35S:DREB1A* Plants

We compared the metabolite profiles of the eight analyzed plants by principal component analysis (PCA; Fig. 2A; Supplemental Tables S2–S6). The cumulative contribution ratio of the PCA reached 61.4% up to the second principal component (PC2), indicating correct application of PCA to the metabolite profiles of the analyzed plants. The PCA showed the following. First, the first principal component (PC1) reflected increases of metabolites in the eight kinds of plants. The PC1 value of plants exposed to 3 d of dehydration was the highest in the eight kinds of plants. Plants exposed to 4 d of cold had the second highest PC1 value. The PC1 value of untreated plants was similar to that of control plants, and that value was the lowest in the eight kinds of plants. Second, PC2 reflected differences in the variety of metabolites according to different environmental conditions. The PC2 value of cold-exposed plants was positive, but PC2 for dehydration-exposed plants was negative. The PC2 values of both untreated and control plants were nearly zero. Third, the metabolite profile of the *35S:DREB1A* plants was similar to that of cold-exposed plants, and the PC2 values of the plants were positive. Fourth, the metabolite profile of *35S:DREB2A-CA* plants was similar to that of dehydration-exposed plants, and PC2 values were negative.

We selected representative metabolites for which the eigenvector values were the first and second highest or lowest, and those values are displayed in bar charts (Fig. 2B). The PC1 eigenvector value of metabolite 190 was the highest. The level of metabolite 190 increased significantly in plants exposed to 3 d of dehydration or 4 d of cold. This metabolite was also detected in *35S:DREB1A* plants but not in *35S:DREB2A-CA* plants. Raffinose had the second highest PC1 eigenvector value. The level of raffinose increased significantly in plants exposed to 3 d of dehydration or 4 d of cold and in *35S:DREB1A* plants. The level of raffinose in the *35S:DREB1A* plants was the highest of those in the eight kinds of plants. We did not detect raffinose in either untreated or control plants. Raffinose was detectable in *35S:DREB2A-CA* plants, but its level was very low compared with *35S:DREB1A* plants. Metabolite 70 had the highest PC2 eigenvector value. The level of metabolite 70 increased significantly in both cold-exposed and *35S:DREB1A* plants but not in dehydration-exposed or *35S:DREB2A-CA* plants. Metabolite 73 had the second highest PC2 eigenvector value. The level of metabolite 73 increased significantly in cold-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants. Metabolite 72 had the lowest PC2 eigenvector value. Metabolite 72 increased significantly in plants exposed to 3 d of dehydration and decreased significantly in both cold-exposed and *35S:DREB1A* plants. Metabolite 4 had the second lowest PC2 eigenvector. The level of metabolite 4 increased significantly in plants exposed to 3 d of dehydration.

To identify candidates that play vital roles under low-temperature conditions, we tried to select metabolites that were unique to both cold-exposed and *35S:DREB1A* plants but were not affected in *35S:DREB2A-CA* plants. Seventeen such metabolites were selected, and their values are displayed in bar charts (Fig. 2, B and C). Among the 17 metabolites, 13 are unknown and four are myoinositol (metabolite 247), galactinol (metabolite 239), raffinose (metabolite 251), and Suc (metabolite 253). The levels of metabolites 55, 70, 92, 155, 156, and 189 increased significantly in both cold-exposed and *35S:DREB1A* plants but not in dehydration-exposed plants. The levels of raffinose, galactinol, myoinositol, Suc, and metabolites 19, 80, 85, 100, 193, 200, and 209 increased significantly in not only cold-exposed and *35S:DREB1A* plants but also in dehydration-exposed plants. Among the 17 metabolites, raffinose, galactinol, myoinositol, Suc, and metabolite 70 were detectable in *35S:DREB2A-CA* plants at very low levels; however, the other 12 metabolites were undetectable in *35S:DREB2A-CA* plants.

Identification of Cold- or Dehydration-Responsive Genes and Downstream Genes of DREB1A and DREB2A

To identify cold- or dehydration-responsive genes and downstream genes of DREB1A and DREB2A, we performed array analyses using an Agilent Arabidopsis 2 Oligo Microarray. In the cold-exposed, dehydration-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, 945, 2,641, 259, and 373 genes were significantly up-regulated, respectively (FDR; $P < 0.05$ and fold change [FC] > 2; Supplemental Tables S7–S12). In contrast, 732, 2,999, 174, and 234 genes were down-regulated significantly in the cold-exposed, dehydration-

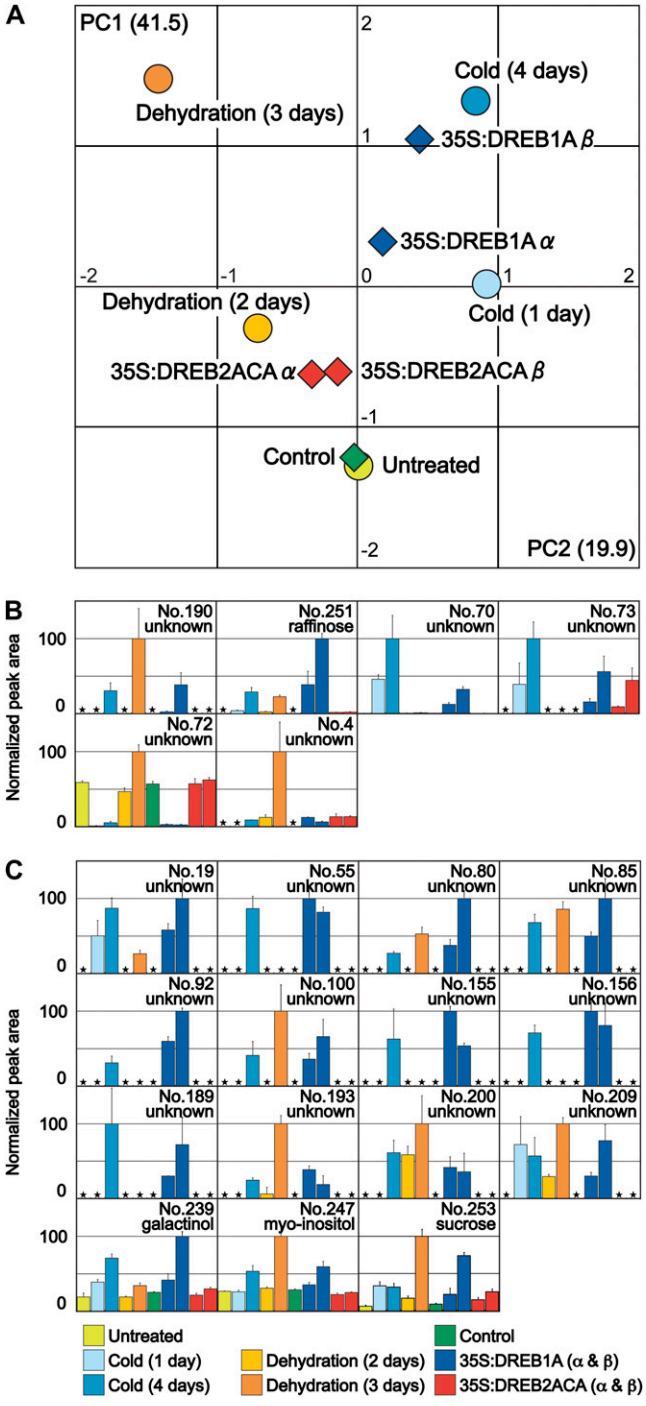

Figure 2. Statistical analyses of metabolite profiles. We analyzed two independent lines of *35S:DREB1A* (α and β) and *35S:DREB2A-CA* (α and β) plants. The levels of metabolites for both DREB1A and DREB2A in each β line were higher than those in each α line. A, PCA of metabolites. The *y* and *x* axes represent PC1 and PC2, respectively. The circles indicate untreated, cold-exposed, and dehydration-exposed plants. The diamonds represent control, *35S:DREB1A*, and *35S:DREB2A-CA* plants. B, Representative metabolites for which the eigenvector values were the first and second highest or lowest. In each case, the maximum level of the metabolite was set to 100. Error bars indicate SD for three experiments. A star indicates that the metabolite was not detected. Metabolites in the α and β lines of each transgenic plant are shown by the left and right bars, respectively. C, Selected metabolites that were increased in both cold-exposed and *35S:DREB1A* plants but minimal in *35S:DREB2A-CA* plants.

exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively (FDR; $P < 0.05$ and FC $<$ ½; Supplemental Tables S7–S12). Of the 259 up-regulated genes in *35S:DREB1A* plants, 125 were unknown and the other gene products included dehydrin, LEA protein, starch-degrading enzymes, transcription factors, and protein kinases. Of the 373 up-regulated genes in *35S:DREB2A-CA* plants, 158 were unknown and the other gene products included dehydrin, LEA protein, HS protein family, enzymes for toxin catabolic processes, transcription factors, and protein kinases (Fig. 3).

Expression Patterns of Genes for Starch-Degrading Enzymes and Sugar Alcohol Synthases in *35S:DREB1A* Plants Were Similar to Those in Plants Exposed to Cold for 4 d

We reconstructed a metabolic enzyme database for Arabidopsis. First, we downloaded all of the amino acid sequence data from The Arabidopsis Information Resource and searched enzyme motifs using the HMMER program (Eddy, 1998). Then, we downloaded data for enzymes involved in metabolism from KEGG, AraCyc, and Kappa-view and performed similarity searches of the downloaded data against all Arabidopsis proteins using the PSI-BLAST program. Based on these results, we selected 2,232 metabolic enzymes from all Arabidopsis proteins (Supplemental Table S13) as a database. Among the genes for the selected 2,232 enzymes, 2,170 were detectable in the microarray (Supplemental Table S13). Of the 2,170 genes, 115, 385, 42, and 44 were significantly up-regulated in cold-exposed, dehydration-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively (FDR; $P < 0.05$ and FC > 2; Supplemental Fig. S4; Supplemental Tables S14–S19). In contrast, 143, 414, 35, and 50 genes were down-regulated significantly in cold-exposed, dehydration-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively (FDR; $P <$ 0.05 and FC $<$ ½; Supplemental Fig. S4; Supplemental Tables S14–S19).

Expression levels of genes encoding starch-degrading enzymes, Suc metabolism enzymes, and sugar alcohol synthases correlated positively with the accumulation of monosaccharides, disaccharides, trisaccharides, and sugar alcohols in cold- and dehydration-exposed plants (Fig. 4). Genes encoding starch-degrading enzymes, such as α-amylase, β-amylase, and glucan-water dikinase, were up-regulated significantly under both cold and dehydration conditions. The downstream products of these enzymes, such as Glc, Fru, and Suc, accumulated under both cold and dehydration conditions. In addition, genes for galactinol synthase were up-regulated significantly under both cold and dehydration conditions. The levels of both galac-

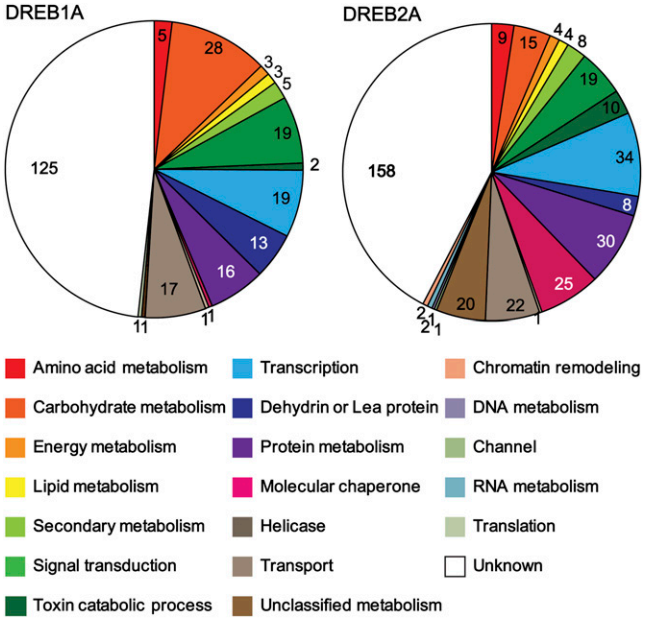

Figure 3. Functional categorization of DREB1A and DREB2A-CA downstream genes. Shown are 20 functional categories of DREB1A and DREB2A-CA downstream genes.

tinol and raffinose increased under both cold and dehydration conditions. On the other hand, these sugars except Glc and Fru increased in the *35S:DREB1A* plants, but none of them increased in the *35S:DREB2A-CA* plants as compared with control plants (Fig. 4).

We then focused on genes for starch-degrading enzymes and sugar alcohol synthase (Fig. 5A). We confirmed expression levels of these genes using quantitative reverse transcription (qRT)-PCR (Fig. 5B). The genes for starch-degrading enzymes were divided into two groups: one included cold-inducible genes, and the other included dehydration-inducible genes. Expression of *AMY3* and *BAM3/BMY8* increased specifically under cold conditions. In contrast, expression of *AMY2*, *BAM1/BMY7*, *BAM2/BMY9*, *BAM6*, and *BAM9/BMY3* increased specifically under dehydration conditions. Furthermore, most of these genes were regulated oppositely under dehydration and cold conditions (Fig. 5). Whereas expression of *AMY3*, *BAM3/BMY8*, *PHS1/PHO1*, *PHS2/PHO2*, *ISA3*, *DPE1*, *DPE2*, and *GWD1/SEX1* increased under cold conditions, these transcripts decreased under dehydration conditions. In addition, expression of *BAM1/BMY7*, *BAM6*, and *BAM9/BMY3* increased under dehydration conditions but decreased under cold conditions. Within the galactinol synthase gene family, expression of *AtGolS3* and *AtGolS2* increased significantly in cold- and dehydration-exposed plants, respectively (Fig. 5). Expression patterns of genes for starch-degrading enzymes and sugar alcohol synthase in *35S:DREB1A* plants were similar to those in plants exposed to cold for 4 d (Fig. 5). However, expression of these genes did not increase in *35S:DREB2A-CA* plants, except for *AtGolS3* and *AtGolS2* (Fig. 5).

DISCUSSION

Previously, we showed that overexpression of *DREB1A* improved both dehydration and freezing stress tolerance, whereas overexpression of *DREB2A-CA* improved dehydration stress tolerance but not freezing tolerance in transgenic plants (Sakuma et al., 2006a). In this study, we used microarrays to analyze genes that are regulated by DREB1A and DREB2A under the same conditions as used for metabolite analyses and found that 259 and 373 genes were sig-

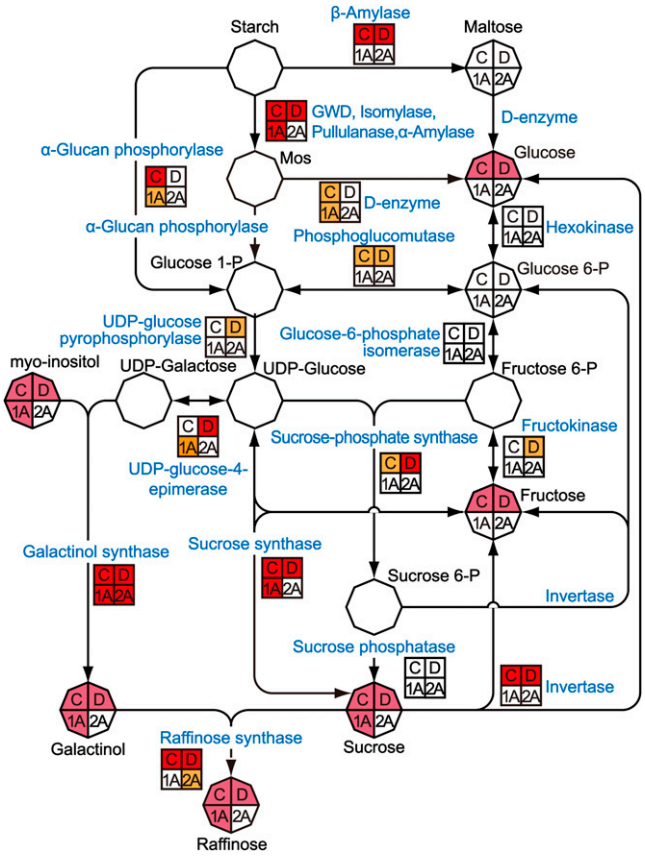

Figure 4. Map of starch degradation and Suc metabolism pathways. Each small square indicates the expression level of the gene that shows the highest expression in each gene family. Red squares show more than 4-fold increase relative to the control. Orange squares show between 2- and 4-fold increase. Octagons indicate metabolite accumulations. Each pink section indicates increased metabolites in each plant. We could not measure starch, maltooligosaccharides, Glc-1-P, Fru-6-P, Suc-6-P, UDP-Glc, and UDP-Gal. C, D, 1A, and 2A indicate cold-exposed, dehydration-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively. We analyzed two independent lines of *35S:DREB1A* (α and β) and *35S:DREB2A-CA* (α and β) plants. GWD, Glucan-water dikinase.

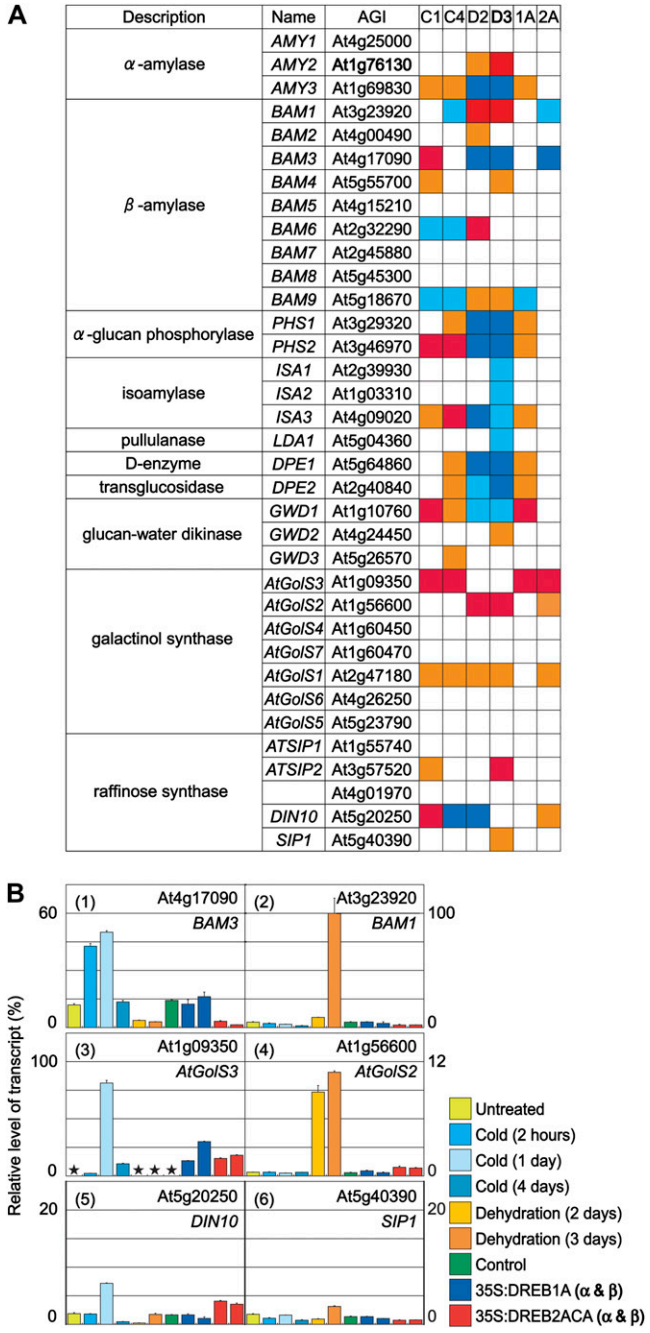

Figure 5. Expression of genes for starch degradation- and Suc metabolism-related enzymes. A, Each small square indicates the level of gene expression. Red squares show more than 4-fold increase relative to the control. Orange squares show between 2- and 4-fold increase. Light blue squares show between ½- and ¼-fold increase. Dark blue squares show less than ¼-fold increase. C1, C4, D2, D3, 1A, and 2A indicate cold-exposed (1 d), cold-exposed (4 d), dehydration-exposed (2 d), dehydration-exposed (3 d), *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively. We analyzed two independent lines of *35S:DREB1A* (α and β) and *35S:DREB2A-CA* (α and β) plants. The levels of *BAM3*, *BAM1*, *AtGolS3*, *AtGolS2*, *DIN10*, and *SIP* transcripts were detected by qRT-PCR. B, The levels of transcripts for genes encoding β-amylase, galactinol, and raffinose synthase determined by qRT-PCR. Panels 1 and 2 show the levels of transcripts for *BAM3* and *BAM1* encoding β-amylase, respectively. Panels 3 and 4 show the levels of transcripts for *AtGolS3* and *AtGolS2* encoding galactinol synthase, respectively. Panels 5 and 6 show the levels of transcripts for *DIN10* and *SIP1* encoding raffinose synthase, respectively. Error bars indicate SD for three experiments. A star indicates that the transcript was not detected.

nificantly increased in *35S:DREB1A* and *35S:DREB2A-CA* plants, respectively, in comparison with vector control plants (Fig. 1). The putative functions of most of the DREB1A- and DREB2A-targeted genes were similar, but certain genes were specific to DREB2A, such as molecular chaperones and enzymes for toxin catabolic processes. We also found that most putative functions of DREB1A downstream gene products were included in those of the DREB2A downstream gene products, but the number of DREB1A downstream genes encoding enzymes for carbohydrate metabolism was much higher than that of DREB2A downstream genes. Thus, expression of these DREB1A-specific downstream genes encoding enzymes for carbohydrate metabolism may be involved in freezing tolerance in *35S:DREB1A* plants.

We aimed to detect many kinds of metabolites that are regulated by abiotic stress using GC-TOF-MS, LC-IT-MS, and CE-MS. GC-TOF-MS and LC-IT-MS are suitable for analysis of polar metabolites and secondary metabolites, respectively. CE-MS was used to analyze charged metabolites. We identified 254 independent metabolites using GC-TOF-MS and CE-MS. Among them, 61.0%, 63.7%, 14.5%, and 11.0% of metabolites increased significantly in cold-exposed, dehydration-exposed, *35S:DREB1A*, and *35S:DREB2A-CA* plants, respectively (Fig. 1). Detected metabolites were compared by PCA (Fig. 2A). The metabolite profiles were classified into three groups according to plant growth conditions: cold, dehydration, and untreated/control. The metabolite profile of the *35S:DREB1A* plants was similar to that of the cold class but not to that of the dehydration class. The metabolite profile of the *35S:DREB2A-CA* plants resembled that of the dehydration class more than that of the cold class. Because *35S:DREB1A* plants, but not *35S:DREB2A-CA* plants, were freezing tolerant, the metabolites for which the level increased in the cold-exposed and *35S:DREB1A* plants but not in the *35S:DREB2A-CA* plants probably participate in improvement of freezing stress tolerance. Seventeen metabolites were selected as candidates. Among them, 13 are unknown and four are myoinositol, Suc, galactinol, and raffinose. Although both transgenic plants showed strong dehydration tolerance, the metabolite profiles in these plants were very different. These results suggest that the dehydration-specific metabolites appearing on the negative side of the PC2 axis may not be important for dehydration-specific tolerance in *35S:DREB1A* plants. Several unknown metabolites that increased under both cold and dehydration conditions and also in both transgenic plants might be involved in dehydration tolerance in the transgenic plants (Supplemental Table S5). We

determined using LC-IT-MS that accumulation of only three secondary metabolites increased significantly in the cold-exposed and *35S:DREB1A* plants (Supplemental Fig. S3). These secondary metabolites also increased in the *35S:DREB2A-CA* plants but not in the dehydration-exposed plants. Korn et al. (2008) also reported that accumulation of several secondary metabolites similar to these metabolites increased in cold-treated Arabidopsis plants. However, our comparative analyses suggest that these secondary metabolites may not be important for freezing tolerance in *35S:DREB1A* plants, because they also increased significantly in the *35S:DREB2A-CA* plants.

Our integrated analysis of metabolites and transcripts indicated that expression of many genes encoding starch-degrading enzymes, Suc metabolism enzymes, and sugar alcohol synthases changed dynamically and resulted in the accumulation of monosaccharides, disaccharides, trisaccharides, and sugar alcohols, including Suc, myoinositol, galactinol, and raffinose, in cold-exposed and dehydration-exposed plants. Because most of these genes are targets of DREB1A, expression of these genes also changed dynamically and the metabolites also accumulated in the *35S:DREB1A* plants. In contrast, because these genes are not regulated by DREB2A (except the genes for galactinol synthase and raffinose synthase), none of these metabolites accumulated in *35S:DREB2A-CA* plants (Fig. 4). Expression of these genes and accumulation of these metabolites correlated with differences in freezing tolerance between the *35S:DREB1A* and *35S:DREB2A-CA* plants. These results suggest that dynamic transcriptional regulation of the carbohydrate network is necessary for the accumulation of specific carbohydrates, such as Suc, galactinol, myoinositol, and raffinose, and that the accumulation of these carbohydrates may be important for improving freezing tolerance in transgenic plants overexpressing *DREB1A*. Moreover, it is likely that DREB1A is one of the major factors regulating carbohydrate metabolism under cold conditions, whereas DREB2A is not.

A cold-inducible galactinol synthase, *AtGolS3*, plays a key role in the accumulation of galactinol and raffinose under cold conditions (Taji et al., 2002; Cook et al., 2004). Although the level of the *AtGolS3* transcript in the *35S:DREB2A-CA* plants was similar to that in cold-exposed and *35S:DREB1A* plants, galactinol and raffinose content in the *35S:DREB2A-CA* plants did not increase compared with cold-exposed and *35S:DREB1A* plants (Figs. 4 and 5). Dynamic changes in gene expression may be necessary for plants to accumulate these metabolites, and strong freezing stress tolerance of *35S:DREB1A* plants may depend on the accumulation of these metabolites. It has been reported that an increase of the raffinose content in transgenic petunia (*Petunia hybrida*) plants improves freezing tolerance (Pennycooke et al., 2003). By contrast, using transgenic Arabidopsis, Zuther et al. (2004) determined that raffinose is not an essential component of basic freezing tolerance and cold acclimation. Future studies may be necessary to establish the importance of raffinose in the freezing tolerance of Arabidopsis plants. Recently, Nishizawa et al. (2008) reported that galactinol and raffinose act as scavengers of the hydroxyl radical in vitro. Under low-temperature conditions, raffinose probably acts not only as an osmoprotectant and a stabilizer of cellular membranes but also as a scavenger of reactive oxygen species to protect the photosynthesis complex in chloroplasts of the cold-exposed and *35S:DREB1A* plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Both wild-type and transgenic Arabidopsis (*Arabidopsis thaliana*) plants were grown in plastic pots filled with peat moss for 3 weeks (principal growth stage 1.07–1.08) under a 16-h-light/8-h-dark regimen (40 $\pm$ 10 μmol photons m^{-2} s^{-1}) at 22°C. The light intensity was the same at 22°C and 4°C. Plants were harvested 2 h later after lights were turned on. For cold treatment, 3-week-old plants were transferred from 22°C to 4°C and were grown for 1 or 4 d. For dehydration treatment, 3-week-old plants were grown for 2 or 3 d without watering. To obtain accurate results, we carefully raised single plants in petri dishes, each containing an equal amount of soil. Soil moisture contents were calculated from soil dry weight. Untreated, the soil moisture content was 84.3%. Under dehydration, on the 2nd d, the soil moisture content was 51.1%. Under dehydration, on the 3rd d, the moisture content was 11.6% (Supplemental Fig. S5).

Metabolite Extraction and Detection

GC-TOF-MS Analysis

Metabolites were extracted from aerial parts of Arabidopsis plants (50 mg) with methanol. Extraction and derivatization were performed as described (Kusano et al., 2007). Prepared metabolites were detected by an Agilent 6890 GC apparatus (Agilent Technologies) coupled to a TOF-MS device (Leco) on a 30-m DB-17ms column (J&W Scientific; 0.25 mm i.d., 0.25-mm film). Ribitol was used as an internal standard (Kusano et al., 2007). The reproducibility of GC-TOF-MS analysis was determined using three biological replicates in each experiment.

CE-MS Analysis

Metabolites were extracted from aerial parts of Arabidopsis plants (50 mg) with chloroform/methanol solution using a mixer and a 5-kD cutoff filter (Millipore). Prepared metabolites were detected by CE-MS (Agilent 1100 series MSD mass spectrometer, Agilent 1100 series isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-MS sprayer kit). Met sulfone was used as an internal standard. The reproducibility of CE-MS analysis was determined using three biological replicates in each experiment. Unknown metabolites were measured by CE-MS.

LC-IT-MS Analysis

Metabolites were extracted from aerial parts of Arabidopsis plants (50 mg) with methanol:acetic acid:water (9:1:10) as described (Tohge et al., 2005). Prepared metabolites were detected by an Agilent HPLC 1100 series coupled to a Finnigan LTQ mass spectrometer (Thermo Electron). Kaempferol 3-*O*-rutinoside was used as a reference compound for the standard curve. The reproducibility of LC-IT-MS analysis was determined using five biological replicates in each experiment.

Microarray and qRT-PCR Analyses

Total RNA was isolated from aerial parts of Arabidopsis plants with Trizol reagent (Invitrogen) and used for the preparation of Cy5- and Cy3-labeled complementary RNA probes. All microarray experiments, including the data

analysis, were performed according to Agilent methods. The reproducibility of microarray analysis was assayed by biological and technical (dye-swap) replicates in each experiment (Invitrogen), and qRT-PCR was performed as described (Sakuma et al., 2006a). Microarray analysis and data mining were carried out as described (Qin et al., 2008). Array design and data from this article have been deposited at MIAMExpress under accession numbers E-MEXP-2173, E-MEXP-2174, and E-MEXP-2175.

Functional Categorization of Genes

Cold- or dehydration-responsive genes and downstream genes of DREB1A and DREB2A were annotated according to results of motif (HMMER) and similarity (PSI-BLAST) searches and were classified according to Gene Ontology annotation at The Arabidopsis Information Resource, KEGG, and Kappa-view.

Supplemental Data

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

Supplemental Figure S1. Venn diagrams of identified metabolites.

Supplemental Figure S2. LC-IT-MS analysis.

Supplemental Figure S3. Functional categorization of DREB1A and DREB2A-CA downstream genes.

Supplemental Figure S4. Venn diagrams of metabolite-related gene expression.

Supplemental Figure S5. Dehydration treatment.

Supplemental Table S1. Mean and SD.

Supplemental Table S2. Sum mean SD.

Supplemental Table S3. Variance covariance matrix.

Supplemental Table S4. Eigenvalue.

Supplemental Table S5. Eigenvector.

Supplemental Table S6. PC score.

Supplemental Table S7. Cold-exposed plants (1 d), all.

Supplemental Table S8. Cold-exposed plants (4 d), all.

Supplemental Table S9. Dehydration-exposed plants (2 d), all.

Supplemental Table S10. Dehydration-exposed plants (3 d), all.

Supplemental Table S11. 35S:DREB1A transgenic plants, all.

Supplemental Table S12. 35S:DREB2A-CA transgenic plants, all.

Supplemental Table S13. List of metabolic enzymes.

Supplemental Table S14. Cold-exposed plants (1 d), metabolic enzymes.

Supplemental Table S15. Cold-exposed plants (4 d), metabolic enzymes.

Supplemental Table S16. Dehydration-exposed plants (2 d), metabolic enzymes.

Supplemental Table S17. Dehydration-exposed plants (3 d), metabolic enzymes.

Supplemental Table S18. 35S:DREB1A transgenic plants, metabolic enzymes.

Supplemental Table S19. 35S:DREB2A-CA transgenic plants, metabolic enzymes.

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