Analysis of Differential Expression Patterns of mRNA and Protein During Cold-acclimation and De-acclimation in *Arabidopsis*[*][S]

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Overwintering plants are capable of exhibiting high levels of cold tolerance, which is acquired through the process of cold acclimation (CA). In contrast to CA, the acquired freezing tolerance is rapidly reduced during cold de-acclimation (DA) and plants resume growth after sensing warm temperatures. In order to better understand plant growth and development, and to aid in the breeding of cold-tolerant plants, it is important to decipher the functional mechanisms of the DA process. In this study, we performed comparative transcriptomic and proteomic analyses during CA and DA. As revealed by shotgun proteomics, we identified 3987 peptides originating from 1569 unique proteins and the corresponding mRNAs were analyzed. Among the 1569 genes, 658 genes were specifically induced at the transcriptional level during the process of cold acclimation. In order to investigate the relationship between mRNA and the corresponding protein expression pattern, a Pearson correlation was analyzed. Interestingly, 199 genes showed a positive correlation of mRNA and protein expression pattern, indicating that both their transcription and translation occurred during CA. However, 226 genes showed a negative correlation of mRNA and protein expression pattern, indicating that their mRNAs were transcribed during CA and were stored for the subsequent DA step. Under this scenario, those proteins were specifically increased during DA without additional transcription of mRNA. In order to confirm the negative correlation of mRNA and protein expression patterns, qRT-PCR and western blot analyses were performed. Mitochondrial malate dehydrogenase 1 (mMDH1) exhibited a negative correlation of mRNA and protein levels, which was characterized by CA-specific mRNA induction and protein accumulation specifically during DA. These data indicate that the expression of specific mRNAs and subsequent accumulation of corresponding proteins are not always in accordance under low temperature stress conditions in plants. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M114.039081, 3602–3611, 2014.

Low temperature stress is one of the major environmental stress factors that affect plant distribution, growth, and productivity. It is well-known that overwintering plants are capable of adapting to cold temperatures via an orchestrated expression of several sets of genes (1–4). This process is termed as cold acclimation (CA)[1] and through this mechanism, plants are capable of acquiring freezing tolerance (5, 6). Especially, overwintering plants acquire freezing tolerance and are capable of surviving under persistent freezing conditions. Previous studies have demonstrated that CA is a complex process that is comprised of multiple steps such as the alteration of membrane fluidity and lipid composition, accumulation of compatible solutes and the regulation of gene expression via the DREB/CBF regulon (1–3, 7–9). Plants are also known to suspend growth under stress conditions as an adaptive measure to avoid damage from freezing temperature. In relation to the observed effect of growth suspension under cold conditions, overexpression of DREB/CBF, which is an important transcription factor for freezing tolerance, exhibits growth retardation under normal conditions (10, 11).

In contrast to CA, cold de-acclimation (DA) is an important regulatory mechanism as a result of the perception of warm

temperatures that enables overwintering plants to resume growth in Spring. In comparison to CA, the DA response is more rapid under both field and experimental conditions. In the case of *Solanum* species, 15 days are required in order to acquire maximum cold tolerance during CA, however, this acquired cold tolerance is lost within only 1 day during DA (12). Similarly, cabbage seedlings have also been reported to lose freezing tolerance and to decrease sugar contents within 24 h of DA (13). In order to better understand the interrelated processes of freezing tolerance and plant growth and development, it is important to decipher the functional mechanisms of regulated gene and protein expression during CA and DA.

RNA regulation is a highly coordinated mechanism that functions to directly affect and regulate the cellular levels of mRNAs and proteins. Previous studies have investigated the global changes of stabilization and/or degradation of mRNA in plants (14, 15). These RNA regulatory mechanisms were shown to be controlled by RNA particles such as stress granules (SGs) and processing bodies (PBs) that are involved in mRNA stabilization and degradation, respectively (16, 17). Functional analyses with defective mutants for various components of these particles revealed sensitive phenotypes under abiotic stress conditions and confirmed the association of these particles to abiotic stress tolerance (18, 19). These data indicate that the regulatory processes of RNA, such as mRNA stabilization or degradation, are important functional components for plant stress responses.

In the case of CA in plants, many cold-inducible mRNAs have been identified by transcriptome analyses with microarray (2, 4). In addition, cold-inducible proteins have also been identified with proteome analyses using 2D electrophoresis or high throughput shotgun proteomics (20–22). However, the resultant data from these two approaches are not completely concordant with one another. It is also known that gene and protein expression patterns might not be identical because of post-transcriptional or translational regulation (23, 24). As a result, we hypothesized that a specific type of RNA regulation, such as post-transcriptional or translational regulation, occurs in plants in response to stress. During CA, many cold-inducible mRNAs are transcribed, however, not all of these transcripts are translated. Some mRNAs are degraded and others are stored in an untranslated state for anticipatory preparation to subsequent environmental alterations. These stored mRNAs are expressed during CA but their corresponding proteins are not translated during CA and are saved for subsequent translation at a later point in time (23, 24).

In order to identify the specific regulatory targets during the processes of CA and DA, we performed comparative transcriptomic and proteomic analyses during CA and DA. The present study revealed several putative targets of translational regulation under low temperature stress in plants. The identification of these targets provides a foundation of evidence to enable us to more completely understand the mechanism of RNA regulation in CA and DA in plants.

EXPERIMENTAL PROCEDURES

Plant Materials, Growth Conditions, and Cold Treatment—*Arabidopsis thaliana* ecotype Columbia-0 was used as the plant source material for the majority of the experiments. Plants were grown on Murashige and Skoog medium plates containing a final concentration of 1% sucrose at a normal growth temperature of (22 °C) under long day (16h/8h light/dark) conditions at $50–75$ μmolm^{-2}s^{-1}. Two week-old *Arabidopsis* samples (NA, nonacclimated) were treated with cold (2 °C) for 7 days (CA7d) under 12 h/12 h light/dark conditions at $75–100$ μmolm^{-2}s^{-1}. Subsequent to cold treatment, DA-treated samples (DA6h, DA12h, and DA24h) were grown at 22 °C for 24 h under long day conditions and samples were harvested at five time points; NA, CA7d, DA6h, DA12h, and DA24h for further experiments.

Plate-based Freezing Tolerance Analysis—A temperature controlled assessment of freezing tolerance was performed essentially as previously described (9, 25) under dark conditions. Plates containing NA, CA, or DA-treated *Arabidopsis* plants were pretreated at -2 °C for 2 h. Plates were nucleated for freezing by dipping the edge of plates into liquid nitrogen. Nucleated plates were subsequently exposed to a controlled freezing program at a cooling rate of (-1 °C/2 h). After reaching desired set-points of sub-zero temperatures, plates were then transferred to 4 °C and thawed overnight in dark conditions. The plates were then returned to normal growth temperature for a period of 2 weeks and the number of surviving plants were counted. A total of three biological replicates were performed.

Shotgun Proteomics—*Arabidopsis* plants were grown on MS plates and treated for CA and DA as described above with three biological replicates. ^{15}N isotope-labeled standard proteins were prepared in order to normalize each sample. Standard plants were grown in ^{15}N substituted MS medium using a stable isotope of ^{15}N of KNO_3 and NH_4NO_3 (SI science, Saitama, Japan) and treated for CA and DA. Total proteins were extracted from CA and DA-treated samples (three biological replicates) using extraction buffer (10 mM Tris-HCl pH 9.0 containing 8 M of urea). Details of sample preparation and nano-LC-MS/MS analysis database searching were previously described (26). Protein samples were digested by trypsin after reduction with dithiothreitol and alkylation with iodoacetamide. Desalted peptide samples were analyzed with a LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) that was coupled with a Dionex Ultimate3000 pump and an HTC-PAL autosampler (CTC analytics, Zwingen, Switzerland). A self-pulled needle (150 mm length $\times$ 100 μm i.d., 6-μm opening) packed with ReproSil C18 materials (3 μm; Dr. Maisch GmbH) was used as an analytical column with a "stone-arch" frit. A spray voltage of 2400 V was applied and the injection volume was 1 μg/5 μl with a flow rate of 500 nL min^{-1}. The mobile phases consisted of 0.5% acetic acid (A) and 0.5% acetic acid and 80% acetonitrile (B). A three-step linear gradient of 5% to 10% B in 5 min, 10% to 40% B in 60 min, 40% to 100% B in 5 min, and 100% B for 10 min was employed. The MS scan range was *m/z* 300 to 1500. The top-10 precursor ions were selected in the MS scan by Orbitrap with resolution = 60,000. Subsequent MS/MS scans were performed with an ion trap in the automated gain control mode, where the automated gain control values of 5.00e + 05 and 1.00e + 04 were set for full MS and MS/MS, respectively. The normalized collision-induced dissociation was set to 35.0. A lock mass function was used for the LTQ-Orbitrap XL in order to obtain constant mass accuracy during the gradient analysis.

Database Searching and Quantitative Analysis—The Mass Navigator version 1.2 (Mitsui Knowledge Industry, Tokyo, Japan) with the default parameters for LTQ-Orbitrap XL was used to create peak lists on the basis of the recorded fragmentation spectra. The *m/z* values of the isotope peaks were converted to the corresponding monoisotopic peaks when the isotope peaks were selected as the precursor ions. In order to improve the quality of MS/MS spectra, the Mass Navigator

software discarded all peaks that were less than 10 absolute intensity and with less than 0.1% of the most intense peaks in the MS/MS spectra. Peptides and proteins were identified by means of automated database searching using Mascot version 2.2 (Matrix Science, London, UK) in The Arabidopsis Information Resource database (TAIR8_pep_20080412, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR8_blastsets/, the number of sequences is 32825) with a precursor mass tolerance of 3 ppm and a fragment ion mass tolerance of 0.8 Da. Furthermore, strict trypsin specificity was employed, which allowed for up to a total of two missed cleavages. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was allowed as a variable modification. Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the "identity" score of each peptide.

For peptide quantification, we used a ^{15}N isotope labeling method as shown in supplemental Fig. S1. ^{15}N isotope-labeled samples were prepared for all five points of the time course samples: NA, CA7d, DA6h, DA12h, and DA24h. A standard sample (which is referred to as "Standard") was prepared by mixing an equal amount of the labeled samples. All nonlabeled samples (which are referred to as "Target") were spiked with an equal amount of the standard sample and analyzed by LC-MS/MS. The peak heights of the identified peptides (Target) and the corresponding ^{15}N isotope-labeled peptide (Standard) pairs in all samples were determined using Mass Navigator v1.2. Relative peptide abundances were calculated by using the peak heights of ^{15}N isotope-labeled peptides as standards. In order to quantify protein expression levels, unique peptides were selected under the conditions where the corresponding peaks were detected in more than 36/45 experiments (three biological and three technical replicates with five time points of samples) after the "Quantitation score" (Mass Navigator) was cut off at 0.7. In order to remove any error from peaks, we assessed the value of "Peak intensity at MSMS triggered retention time", which represents the confidence of peptide peaks. When the value of "Peak intensity (Standard)" was "-1", the "Peak top height (Target)" was calculated as a "nondetected" peak. When the value of the Peak intensity (Target) was "-1", the, Peak top height (Target) was calculated as "0". The average values of peptide "Peak top heights" were calculated in each experiment using the peptide values of "Peak top height (Target)" divided by "Peak top height (Standard)". The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (27) via the PRIDE partner repository with the dataset identifier PXD001216.

Microarray data were utilized for comparative analyses of expression levels of each peptide to respective mRNA's corresponding to those proteins. In order to detect a relationship between mRNA and peptide levels, Pearson product-moment correlation coefficients for each gene were calculated from Z-scores of mRNAs and peptides of all five data points. These Z-scores were separately obtained from mRNA and peptide levels. In the event that multiple peptides were detected from a single protein, peptides with the highest Pearson correlation values were used as relative expression of its protein and for further analysis.

Microarray Analysis—Total RNA was extracted from CA and DA-treated samples (three biological replicates) using the Plant RNA reagents (Invitrogen, Carlsbad, CA). After isolation, RNAs were synthesized into cDNAs using 500 ng of total RNA. cRNA was labeled with a single color (Cy3) using Quick Amp labeling kit (Agilent Technologies, Palo Alto, CA) and hybridized to an *Arabidopsis* oligo DNA microarray Ver. 3.0 (Agilent Technologies). The microarray data are available on the GEO website (GEO ID: GSE49796). Arrays were scanned with a microarray scanner (G2505B, Agilent Technologies) and analyzed using GeneSpring Ver.7 (Agilent Technologies). Raw signals less than 0.01 were adjusted to 0.01 and a 75 percentile normalization was performed for each chip. These data were used for reference data of mRNA expression for corresponding proteins, which was analyzed by shotgun proteomics.

Protein Identification of Spots from 2D-PAGE Gel—CA7d or DA6h protein samples were separated on 2D-PAGE and visualized using a SilverSNAP for Mass Spectrometry kit (Thermo Fisher Scientific). Protein spots were cut out from gels and destained using the same SilverSNAP kit. Details pertaining to the sample preparation were previously described (28) and the analysis and identification of peptides by the LTQ-Orbitrap XL system (Thermo Fisher Scientific) was described above.

qRT-PCR and Western Blot Analysis—Total RNAs were prepared from CA and DA-treated samples (three biological replicates) using the Plant RNA Reagent (Invitrogen) and cDNAs were synthesized using Quantitech cDNA synthesis kit (Qiagen, Venlo, Netherlands). The generated cDNAs were subsequently used as templates for qRT-PCR analyses that were performed with StepOne Plus (Applied Biosystems, Foster City, CA) using a Fast SYBR Green MasterMix (Applied Biosystems). The *ACT2* gene was used as a reference for CA and DA-treated sample analyses because *ACT2* expression was not significantly altered within our microarray analyses under cold and de-acclimated conditions. The following primer sets were used for amplification of *mMDH1*: 5′-TCAACGGTGTTCCAGATGTC-3′ and 5′-CACCTTCGAGGCAAAGAAAG-3′ and *ACT2*: 5′-TGCCAAT-CTACGAGGGTTTCT-3′ and 5′-CTTACAATTTCCCGCTCTGC-3′. Data were subsequently analyzed by StepOne Plus software (Applied Biosystems).

Total proteins were extracted from CA and DA-treated samples and 10 μg of total proteins were separated with SDS-PAGE (29). After transferring to nitrocellulose membrane, Hybond-C (GE Healthcare, Little Chalfont, England), anti-MDH2 antibody (Abcam, Cambridge, MA) and anti-rabbit IgG, which was conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA), were used for the detection of mMDH1. Protein signals were detected using SuperSignal West Pico (Thermo Fisher Scientific) and LAS-4000 (Fuji film, Tokyo, Japan) and image analyses were performed with ImageJ software. Data were analyzed from three biological replicates.

Polysomal Profiling Analysis—A polysomal fraction was prepared using a sucrose density gradient ultracentrifugation method (23, 30). CA and DA-treated seedling samples were ground to a fine powder and 300 μg samples were hydrated in 1 ml of extraction buffer [200 mM Tris pH 9.0 containing 200 mM KCl, 36 mM MgCl$_2$, 25 mM ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 100 mM 2-mercaptoethanol, 50 mg/ml cycloheximide, 50 mg/ml chloramphenicol, 1% (v/v) Triton X-100, 1% (v/v) Brij-35, 1% (v/v) Tween-40, and 1% (v/v) Nonidet P-40]. After removal of cell debris by centrifugation, 1 ml of the supernatant was loaded onto 9 ml of sucrose gradient. Linear 10–50% (w/v) sucrose gradients were prepared according to the methods described by Abe and Davies (31). After centrifugation for 90 min at 4 °C at 40,000 rpm (275,000 × *g*) using a SW41Ti rotor (Beckman Coulter, Brea, CA), the gradient was loaded into Monitor UV-900 (GE Healthcare) for scanning at 254 nm using a perista pump (ATTO, Tokyo, Japan).

RESULTS

Freezing Tolerance of Arabidopsis—Similar to plants such as winter wheat, rye, barley, and spinach; *Arabidopsis thaliana* is also capable of cold acclimation and acquiring significant levels of freezing tolerance. Under our experimental conditions, cold acclimated *Arabidopsis* plants were confirmed to survive at temperatures that were below −12 °C (Fig. 1*A*). At least 3 days of exposure to low temperature conditions were required for *Arabidopsis* to acquire a high level of freezing

A

NA   CA

-8 °C

-10 °C

-12 °C

B

-16 °C

CA7d

DA6h

DA12h

DA18h

DA24h

C
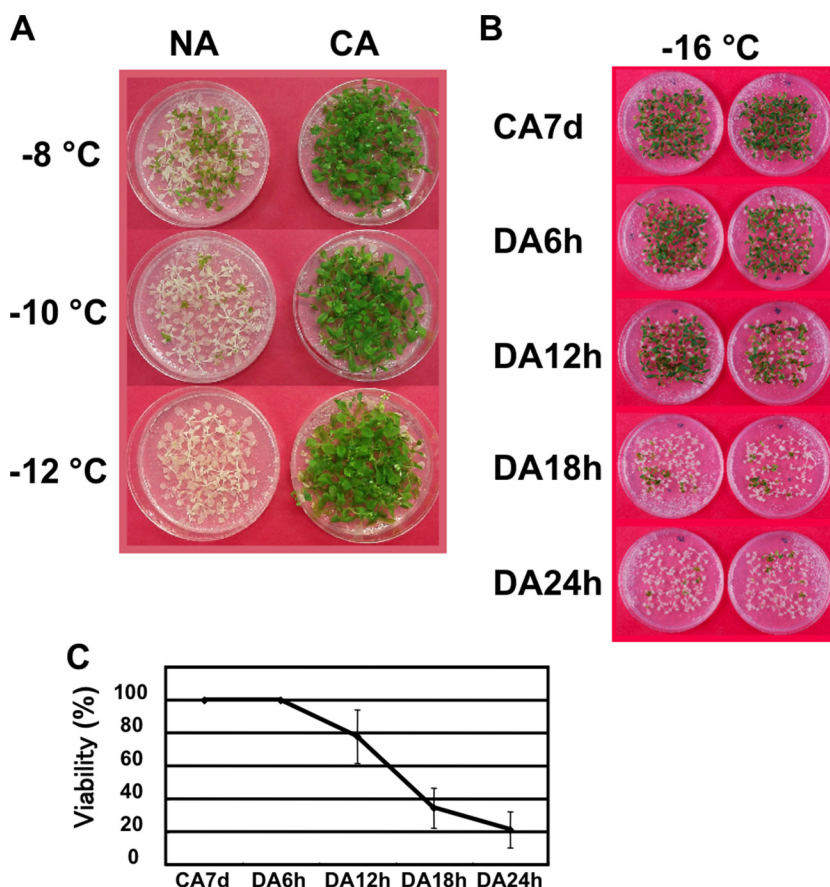
FIG. 1. **Freezing tolerance of *Arabidopsis* plants during nonacclimated, cold-acclimated, and de-acclimated conditions.** *A*, Two week-old nonacclimated (NA) and 7 days cold-acclimated (CA) plants were frozen at −8, −10, and −12 °C or *B*, CA7d. De-acclimated plants (DA6h, DA12h, DA18h, and DA24h) were frozen at −16 °C and then grown at normal temperature for an additional 2 weeks. *C*, Survival rate was measured using a controlled freezing test at −16 °C with three biological replicates.

tolerance. It is known that *Arabidopsis* requires a 1 week period of cold acclimation in order to stimulate a maximum level of freezing tolerance (32). Conversely, when plants were transferred to normal growth temperature conditions after exposure to cold treatments, plants began to rapidly de-acclimate within 24 h under our experimental conditions and freezing tolerance quickly disappeared (Fig. 1*B*, 1*C*). In good accordance to previous studies, these data suggest that *Arabidopsis* is capable of rapidly perceiving warm temperature and quickly transitions away from a CA state (12, 13, 33). In order to better understand the CA and DA mechanism and rapid transitions, we designed a comparative study that incorporated the usage of both CA and DA-treated samples for protein and mRNA analyses.

Protein Expression and Corresponding mRNA Expression Analysis—To understand the gene and protein regulatory mechanism during CA and DA, protein expression analyses were performed with shotgun proteomics. Corresponding mRNAs were analyzed with microarray analyses using protein and RNA that were extracted from CA and DA-treated plant materials. Two week-old seedlings were used as a control (NA) and were then subsequently treated at 2 °C for 7 days (CA7d). For the DA treatment, plants were harvested at 6, 12, and 24 h after exposure to 22 °C (DA6h, DA12h, and DA24h). For transcriptome analyses, DA4h and DA10h samples were

collected and mRNAs were prepared with consideration for the time lag of translation.

According to the shotgun proteomics, 6945 peptides were detected within all of the NA, CA and DA-treated samples and 6307 unique peptides were used for further analysis (supplemental Table S1, annotated MS/MS spectra of the identified peptides were summarized in supplemental Fig. S2). To obtain the expression pattern of mRNA corresponding to the proteins that were identified from the shotgun proteomics approach, we performed microarray analyses with the same CA and DA-treated samples (supplemental Table S2). In order to verify that the experimental conditions were properly implemented for the study, we analyzed the expression patterns for well-known cold-inducible genes (*RD29A*, *COR15a*, and *KIN2*) at both the mRNA and protein level. Results from the analysis of these control genes revealed clear up-regulation in response to low temperature (supplemental Fig. S3) and therefore confirmed that our cold treatment and subsequent transcriptome and proteome analyses were reliable under our experimental conditions. Finally, expression levels of 3987 peptides corresponding 1569 proteins and mRNAs corresponding those protein were used for comparative quantitative analyses during CA and DA (supplemental Table S2).

Identification of Putative Targets of Translational Regulation—To clarify how mRNA expression and the accumulation
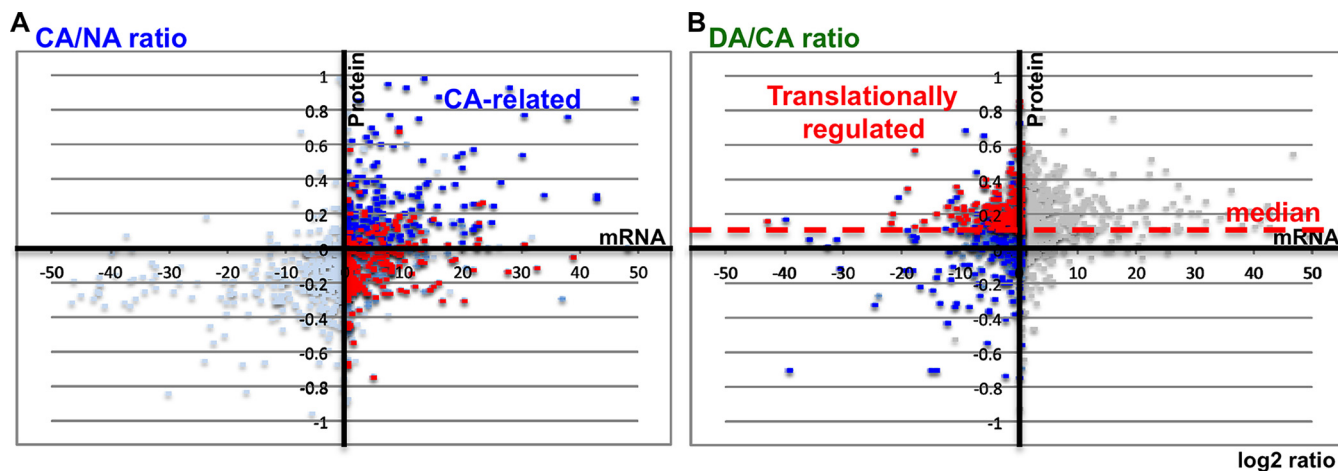
FIG. 2. **Comparative analysis of mRNA expression and protein expression during CA and DA.** mRNA and protein expression patterns were assessed with microarrays and shotgun proteomics, respectively. The $\log_2$ ratio of mRNAs and proteins from CA/NA, *A*, and DA/CA, *B*, were plotted. In the case of mRNA and protein plots, DA4h/CA7d and DA6h/CA7d were calculated, respectively. Horizontal and vertical plots showed alteration of expression patterns for mRNAs and proteins, respectively. Blue spots indicate the genes that showed a positive correlation of mRNAs and proteins on CA/NA and expression patterns of mRNA and protein showed positive correlation during all points of samples. Red spots indicate the genes that showed values greater than the median (red dotted line) of protein but 0 or negative values of mRNA on DA/CA and expression patterns of mRNA and protein showed negative values for all points of samples.

of protein changes during CA and DA, the ratio of CA/NA or DA/CA of mRNA and proteins were calculated and plotted (Fig. 2). Plotting of the CA/NA data revealed alterations of mRNA and protein during CA (Fig. 2*A*) and DA/CA showed these changes early within the DA step (Fig. 2*B*). It was apparent that the ratio values of mRNA and protein expression were different and the mRNA ratio was higher than that of proteins (Fig. 2). This is most likely because of the fact that the induction rate of mRNA and translation speed could not be correlated with each other in all of the cases (34).

In order to identify genes that were specifically induced by low temperature, we focused our analysis on the plots where mRNA levels were up-regulated under CA conditions and down-regulated during DA. Among 1569 genes, 658 genes showed positive values of mRNA in CA/NA (Fig. 2*A*, right half) and negative values of mRNA in DA/CA (Fig. 2*B*, left half); indicating that these mRNAs were specifically expressed during CA. To distinguish between CA-related genes and putative targets of translational regulation, we performed a Pearson correlation analysis of mRNA and protein expression patterns using all points of samples including NA, CA, and DA. These data were summarized and presented in the supplemental Table S2. When both translational data and correlation data were analyzed among 658 cold-inducible genes, only 199 genes were translated in CA and these mRNA and protein expression patterns were positively correlated (Fig. 2, blue spots). Both mRNA and protein expression patterns of blue spots were up-regulated during the NA to CA step (Fig. 2*A*) and down regulated during the CA to DA step (Fig. 2*B*), respectively. In fact, the expression of these 199 genes was specific to CA and their respective proteins also increased during CA. Therefore, it is reasonable to consider that these

are CA-related genes/proteins and that they might possess putative functions for freezing tolerance. In contrast, 226 genes were translated specifically in DA (red spots, Fig. 2*A*, and above median of Fig. 2*B*) and showed a negative correlation between mRNA and protein in CA and DA-treated samples. This result indicated that the mRNA and protein expression patterns of red spots differed between CA and DA samples. To analyze protein levels in DA/CA, the median of all protein expression data was calculated and used instead of the zero line because the translational speed is increased in DA. When the plots of total mRNAs between CA/NA and DA/CA were compared, the medians of the horizontal direction (mRNAs) were similar. However, when we compared the median of the vertical direction (proteins), the DA/CA total plots shifted upward in comparison to CA/NA in our experimental conditions. It is plausible that these data resulted from a downshift of translational speed under cold temperature conditions. Therefore, it might be deduced that total protein synthesis was activated during the CA to DA step but that the synthesis of mRNA was not significantly altered during the same transition period. In order to address the general shift of translation during CA to DA transition, we used the median of ratio (DA/CA) of total detectable protein instead of a zero line to analyze the DA-specific translational targets in this study. These data indicated that these 226 mRNAs were expressed during CA (Fig. 2*A*, red spots) but that their corresponding proteins were expressed during DA (Fig. 2*B*, red spots). According to this comparative analysis, approximately half of the cold-inducible mRNAs were transcribed, translated and functional in CA. However, the other half were transcribed during CA and might be stored for a subsequent step where they gained functionality during DA.

TABLE I

Gene ontology analysis of CA-related and translationally regulated target genes

GO Biological process, functional category[a]	All genes	CA-related genes		Translationally regulated targets	
	Gene count	Gene count	Enrichment[b]	Gene count	Enrichment[b]
Protein metabolism	506	75	1.86E-01	99	1.10E-04
DNA or RNA metabolism	59	6	8.23E-01	14	2.71E-02
Other cellular processes	1176	162	3.65E-01	178	7.81E-02
Other metabolic processes	1147	147	7.65E-01	170	1.45E-01
Response to stress	527	78	1.84E-01	73	5.08E-01
Unknown biological processes	210	15	9.99E-01	29	5.31E-01
Response to abiotic or biotic stimulus	509	77	1.35E-01	68	6.41E-01
Cell organization and biogenesis	426	44	9.81E-01	55	7.32E-01
Other biological processes	448	69	1.16E-01	57	7.75E-01
Signal transduction	123	12	9.15E-01	13	8.85E-01
Transport	323	45	4.19E-01	32	9.88E-01
Transcription, DNA-dependent	109	13	7.21E-01	8	9.88E-01
Developmental processes	318	47	2.57E-01	30	9.94E-01
Electron transport or energy pathways	247	33	5.43E-01	20	9.98E-01

[a] Genes were functionally categorized using GO slim in TAIR.
[b] The p value for overlap was calculated using the hypergeometric distribution.

We performed a gene ontology analysis of CA-related and translationally regulated targets as a means to identify the function of these targets of translational regulation. An enrichment test showed that the genes within the functional category of protein metabolism were significantly enriched in translationally regulated targets (Table I). This category contained ribosomal proteins (46 genes out of 226 genes) and translational initiation factors, such as translation initiation factor 3 subunit H1 (TIF3H1), eukaryotic translation elongation factor 1B (EEF1B), eukaryotic translation initiation factor 4A-2 and 3C (EIF4A-2 and EIF3C) (supplemental Table S3). When the detailed expression patterns of these 46 ribosomal genes were analyzed, mRNAs were induced in CA and decreased in DA. However, their corresponding proteins were not CA-inducible and increased during DA (Fig. 3*A*). In contrast to translationally regulated targets, the expression pattern of CA-related genes such as *RD29A*, *COR15a*, and *KIN2* showed that both mRNAs and proteins were CA-inducible (Fig. 3*B*, supplemental Fig. S3, and supplemental Table S4). These data suggested that translational machinery was translated during an early step of DA and that those mRNA were already prepared in a previous step.

mMDH1 is a Putative Target of Translational Regulation—In addition to the aforementioned experimental analyses, we also compared protein expression patterns using the same time course samples during CA and DA using 2D-DIGE (two dimensional fluorescence difference gel electrophoresis analysis) (supplemental Fig. S4 and supplemental text). Protein spots were quantified by measuring the intensity of fluorescence and 115 protein expression patterns during NA, CA7d, DA6h, DA12h, and DA24h were analyzed. 2D-DIGE analyses revealed that 51 proteins specifically increased during DA and 46 selected protein spots were identified by LC-MS/MS analysis (supplemental Table S5). Corresponding mRNA expression patterns of these identified proteins during CA and DA

were also obtained and analyzed from microarray data. Among the proteins that were identified, mitochondrial malate dehydrogenase1 (mMDH1), aspartate aminotransferase 1 (ASP1), and nucleosome assembly protein 1;2 (NAP1;2) represented interesting target candidates of translational regulation. These candidates were contained in the overall list of targets as described above (supplemental Table S3). Because these genes are related to plant development, it is possible that they might function to rapidly resume growth during DA step. In order to confirm that mMDH1 is indeed a target of translational regulation, we analyzed mMDH1 mRNA and protein expression with qRT-PCR and western blot analyses, respectively. qRT-PCR data revealed that *mMDH1* mRNA expression increased during CA and these data were in good accordance to those obtained by microarray analysis (Fig. 4). For protein expression analysis, a single band of ~35.8 kDa was detected, which was consistent with the calculated molecular weight of mMDH1. Similar to the data obtained from the shotgun proteomics, it was confirmed that the mMDH1 protein accumulated during DA (Fig. 4). Taken together, these data demonstrated that *mMDH1* mRNA was transcribed during CA but its corresponding protein was not concomitantly accumulated; and was subsequently translated during the de-acclimation period. Therefore, we hypothesize that mMDH1 is one of the translationally regulated targets within *Arabidopsis* that may function for rapidly resuming growth in de-acclimated plants.

Polysomal Profiling Analysis in CA and DA—We performed polysomal profiling with sucrose gradient analysis to examine how the translation state was altered during CA and DA. Messenger ribonucleoprotein (mRNP) complexes were separated into subpolysomes, which contain free RNA and 40S, 60S, and 80S complexes, and polysomes or other large complex fractions (supplemental Fig. S5). A subpolysome fraction containing 40S, 60S, and 80S complexes indicates that this
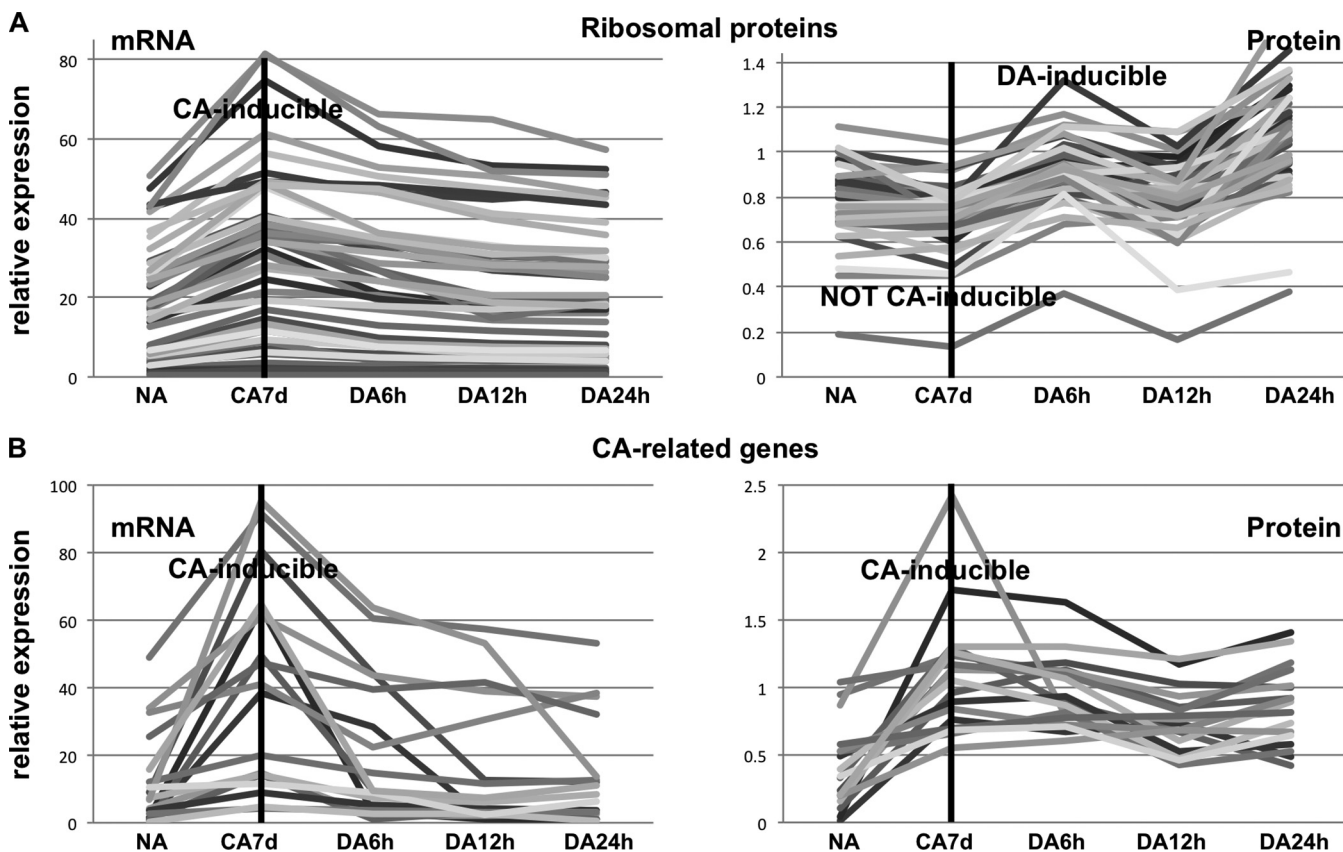
FIG. 3. **Expression patterns of mRNAs and proteins of regulated targets and cold inducible genes during CA and DA.** *A*, Relative expression patterns of ribosomal protein genes and *B*, well-known CA-related genes. Left panel shows mRNA expression patterns and right panel contains protein expression patterns. mRNAs were analyzed using microarray data and proteins were evaluated using shotgun proteomics data.

FIG. 4. **mRNA and protein expression patterns of mMDH1 during CA and DA.** Total RNA was extracted and converted into cDNA to serve as a template for qRT-PCR analyses. mRNA expression was analyzed using qRT-PCR and protein expression was analyzed by western blot analysis. After normalization to *ACT2* mRNA, the relative expression pattern of *mMDH1* was presented (left bottom). Data are shown as the means of three biological replicates. Total protein extracts were separated by SDS-PAGE and signal was detected using a MDH antibody (MDH2, Abcam). Relative expression was analyzed using ImageJ software. Data are shown as the means of three biological replicates. Microarray data (left) and shotgun data (right) are presented in the top panel.
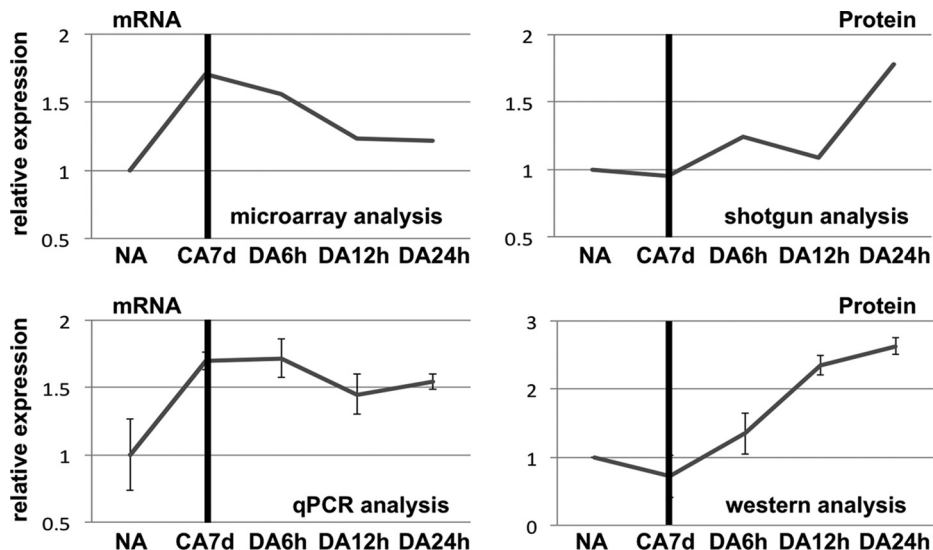
fraction contains the translation initiation step of mRNPs, whose mRNAs were scanned by small subunits of ribosomes and followed by joining the large subunits of ribosomal proteins (Fig. 5). When this subpolysomal fraction was compared in CA and DA, the subpolysome peak decreased in CA5d and

CA7d samples and increased in DA6h and DA12h (Fig. 5). These data indicate that translational initiation was decreasing in CA but increasing again during the early step of DA. On the other hand, the polysomal fraction was accumulated in CA compared with NA or DA6h; indicating that CA-related trans-
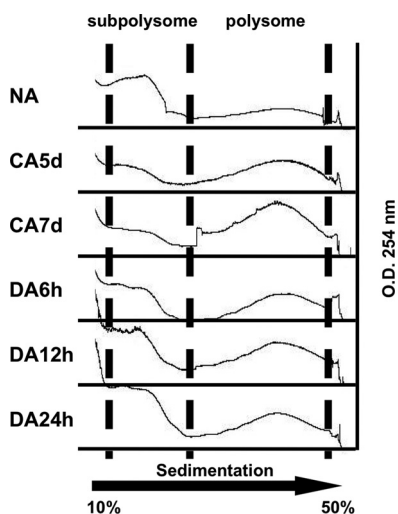
FIG. 5. **Polysomal profiling during CA and DA.** Polysomal fractionation was prepared from NA, CA5d, CA7d, DA6h, DA12h, and DA24h samples and separated through 10–50% sucrose density gradients by ultracentrifugation. Separated complexes were scanned at 254 nm and recorded. The absorbance peaks represent subpolysome containing single ribosome subunits (40S and 60S) or monosomes (80S) and polysome complexes.
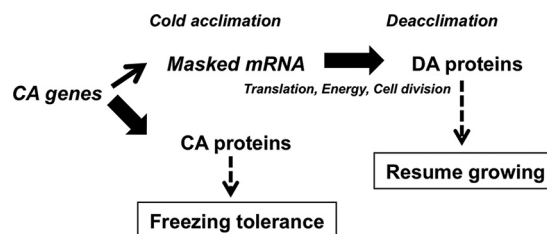

FIG. 6. **Possible model of translational regulation during CA and DA in *Arabidopsis*.** CA genes are translated to CA proteins and have potential functional roles in freezing tolerance. Specific sets of CA genes are masked during CA and translated in the early stage of DA. These specific subsets of genes may have specialized roles for resuming plant growth.

lation was promoted in CA (Fig. 5). Although these data are consistent with previous reports (35, 36), our proteomics data detected approximately the same number of CA-related and translationally regulated targets; indicating that many cold-inducible mRNAs were not translated and stored during CA. Thus, it is possible that this accumulated polysomal peak contains other large complexes such as inactive mRNPs.

DISCUSSION

The process and mechanisms of cold acclimation (CA) have been very well studied at both the physiological and molecular levels; however, the process of de-acclimation (DA) is not as well understood. In order increase our understanding of DA on a molecular level, we designed comparative transcriptome and proteome analyses to characterize the expression profiles of genes and proteins during CA and DA. In this study, we demonstrated that many cold-inducible genes were transcribed and translated, however a similar number of corresponding cold-inducible genes were not translated during the CA stage and were translated at a later stage such as de-acclimation. According to a correlation analysis of mRNA and protein expression patterns, over 200 genes showed negative correlation when their mRNA and protein expression patterns were compared during CA and DA (Fig. 2 and supplemental Table S3). In addition to these gene expression patterns, we identified 286 DA-specific genes, which were translated during DA with *de novo* synthesis of mRNAs (supplemental Table S6). These data imply that these are likely regulated by translational regulation, which alters protein expression patterns in response to environmental alterations or developmental changes. In animals, Y-box binding protein (YB-1) or frog Y-box protein 2 (FRGY2) repress

mRNA translation and enable the storage of mRNAs for subsequent use during embryogenesis or to resume translation by growth stimulation (37, 38). Thus, we hypothesize that these translationally regulated mRNAs were expressed and then stored during CA until the early steps of de-acclimation where they could be rapidly translated for rapid production of corresponding proteins with specific functions during the early stages of the DA process. Because of the disjunction between the up-regulation of specific genes and the lack of corresponding protein accumulation during CA, is likely that the translation of proteins during DA was primed with stored mRNA and occurred without requiring *de novo* mRNA synthesis. Similar regulation was observed in the case of seed germination; stored mRNA were used during germination without requiring *de novo* transcription (39).

Interestingly, approximately half of the cold-inducible genes were not translated during the same period and were stored for the subsequent step. Although these are still candidates of translationally regulated targets, many genes might function for the DA process but were not related to freezing tolerance. It was intriguing that the expression of so many proteins were regulated during the cold response of plants, although we knew from experience that the expression of specific mRNAs and subsequent accumulation of corresponding proteins are not always in accordance. These data suggested that transcriptome analysis only informs the expression level of mRNA and should not be used to predict and inform corresponding levels of proteins under low temperature stress conditions in plants.

We performed an analysis of gene ontology in order to elucidate the types of genes that were stored and function in DA. Because ribosomal proteins and translational initiation factors represented the major targets of translational regulation, it is plausible that they possess important functional roles for the DA process. It is apparent that plants possess mechanisms to enable them to rapidly change from a cold acclimated to a de-acclimated state; a process that is initiated by producing translational machinery during the early steps of DA. In contrast to the de-acclimated state, the mRNA that is required for encoding translational machinery is already produced during cold acclimation (Fig. 6). We hypothesize that

this mechanism facilitates the rapid resumption of normal cellular functions and metabolism during DA. Polysomal profiling of CA and DA samples revealed that the subpolysome fraction increased during early points of the DA process. These data indicate that translational initiation was increased in DA and this may also be the reason why translational machinery was increased at an early point in the DA process. This is supported by the data pertaining to target candidates that encoded genes related to ribosomal proteins and translational initiation factors. mRNAs encoding ribosomal proteins were stored during CA and translated in DA, but additional ribosomal proteins were identified as CA-related genes in our list of candidates (Functional category: protein metabolism) (Table I and supplemental Table S4). Although an enrichment test did not show significant values compared with translationally regulated targets of protein metabolism (Table I), these data support the hypothesis that plants use different sets of translational machinery for various environmental conditions or stress responses.

Comparative analyses of 2D-DIGE and microarrays revealed additional regulated target candidates encoding primary metabolism related enzymes and plant development related proteins such as mMDH1 and NAP1 (Fig. 4 and supplemental Table S3). Previous studies have documented the functional importance of mMDH1 and confirmed that it is essential for plant growth and development. Mutants of *mmdh1* and *mmdh2* showed slow growth and altered photorespiration (40). NAP1 is functionally important for plant growth because of its relation to cell cycles via the regulation of transcription or DNA repair (41, 42). Under stress conditions, plants are known to suspend growing as an adaptive mechanism to avoid damage and to promote the resumption of growth after nonstress conditions are sensed (43, 44). We hypothesize that these regulated targets function to rapidly resume plant growth during DA. As a result, plants appear to possess a mechanism to resume plant growth during DA by providing energy or promoting cell cycles during the early step of the DA process (Fig. 6).

As a result of our comparative analysis, we identified a large number of candidates that are targets of translational regulation. It is likely that the mRNAs were stored within inactive translational complexes. RNA granules, such as stress granules, or RNA masking complexes have been studied for their relationship to RNA regulation with a special emphasis on their roles in mRNA stabilization or storage (45, 46). These stress granules or RNA masking complexes consist of untranslated mRNAs and many RNA-binding proteins. It is thought that the size of the complexes might increase under stress conditions (47, 48). Protein synthesis is also thought to be increasing during CA because of the increased polysomal fraction (36). Although our data also showed that the polysomal fraction increased during CA, it is possible that these large complexes contain large inactive complexes such as a RNA masking system or stress granules; in addition to translationally active polysomes.

In this study, we demonstrated that translational regulation is one of the major steps to determine protein levels under stress conditions in plants and that comparative analysis is a powerful technique to identify the RNA regulation mechanism. Although putative targets of translation regulation were identified in our study, the precise mechanism of regulation remains unclear. Further analyses are required to identify and characterize complexes of stress granules or RNA masking components to better understand their functional roles during the CA and DA processes.

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