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Changes in mRNA Stability Associated with Cold Stress in Arabidopsis Cells

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Control of mRNA half-life is a powerful strategy to adjust individual mRNA levels to various stress conditions, because the mRNA degradation rate controls not only the steady-state mRNA level but also the transition speed of mRNA levels. Here, we analyzed mRNA half-life changes in response to cold stress in Arabidopsis cells using genome-wide analysis, in which mRNA half-life measurements and transcriptome analysis were combined. Half-lives of average transcripts were determined to be elongated under cold conditions. Taking this general shift into account, we identified more than a thousand transcripts that were classified as relatively stabilized or relatively destabilized. The relatively stabilized class was predominantly observed in functional categories that included various regulators involved in transcriptional, post-transcriptional and post-translational processes. On the other hand, the relatively destabilized class was enriched in categories related to stress and hormonal response proteins, supporting the idea that rapid decay of mRNA is advantageous for swift responses to stress. In addition, pentatricopeptide repeat, cyclin-like F-box and Myb transcription factor protein families were significantly over-represented in the relatively destabilized class. The global analysis presented here demonstrates not only the importance of mRNA turnover control in the cold stress response but also several structural characteristics that might be important in the control of mRNA stability.

Keywords: Arabidopsis cultured cells • Cold stress response • mRNA decay array • mRNA half-life • Pentatricopeptide repeat protein • Transcriptome analysis.

Abbreviations: CBF, C-repeat-binding factor; DREB, dehydration response element-binding factor; FunCat, Functional Catalogue; ICE1, inducer of CBF expression 1; NMD, nonsense-mediated mRNA decay; PPR, pentatricopeptide repeat; PPRL, PPR protein-like; qRT–PCR, quantitative reverse transcription–PCR; RMA, Robust Multichip Average; TAIR, the Arabidopsis Information Resource; uORF, upstream open reading frame; UTR, untranslated region.

The microarray data presented in this paper are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE31837.

Introduction

Plants cannot escape from unfavorable environmental conditions; therefore, they have developed various strategies to respond and adjust to biotic and abiotic stresses for their survival. Control of gene expression is an important strategy in the stress response. Cold stress adversely affects the growth and development of plants. Most temperate and subarctic plants have a system to acquire freezing tolerance through pre-exposure to non-freezing low temperatures, known as cold acclimation. As a major transcriptional regulation pathway during cold acclimation, the transcriptional cascade of C-repeat-binding factors (CBFs)/dehydration response element-binding factors (DREBs) has been reported (Shinozaki and Yamaguchi-Shinozaki 2000, Thomashow 2001). CBFs/DREBs are cold-inducible transcriptional activators that bind to *cis*-elements

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in the promoters of cold-responsive genes (Chinnusamy et al. 2007, Zhu et al. 2007, Gorsuch et al. 2010, Thomashow 2010). Expression of CBF3 is controlled by the transcription factor, inducer of CBF expression 1 (ICE1), which is constitutively expressed and activated by cold stress through post-translational modifications such as phosphorylation and sumoylation (Dong et al. 2006, Miura et al. 2007). To understand the role of CBFs/DREBs, many investigators have examined changes in mRNA accumulation in response to cold stress, as well as overexpression of CBFs/DREBs by expression profiling (Fowler and Thomashow 2002, Maruyama et al. 2004, Oono et al. 2006, Matsui et al. 2008, Sasaki et al. 2008). One such transcriptome analysis, which was conducted to identify a robust set of cold-responsive transcripts, revealed that approximately 2% of the transcripts are cold responsive, and CBF2 is involved in the regulation of 28% of them (Vogel et al. 2005, Thomashow 2010). Moreover, the upstream factor, ICE1, is involved in the regulation of 40% of the cold-responsive transcripts (Lee et al. 2005). However, regulatory systems for the remaining cold-responsive transcripts remain unknown.

The amount of individual mRNA is determined by a balance between transcription speed and mRNA degradation rate. Most studies on the control of gene expression have focused on the rate of mRNA synthesis, while few attempts have been made to understand the functional relevance of mRNA stability control. According to the simple kinetics of mRNA turnover, the mRNA degradation rate is important not only for determining the steady-state mRNA level, but also for determining the transition speed of mRNA levels (Perez-Ortin et al. 2007). Several lines of evidence in plants emphasize the importance of mRNA stability control. For example, some genes that are involved in the regulation of methionine, nitrogen and sucrose levels are controlled by mRNA stability (Chiba et al. 1999, Ho et al. 2001, Ortega et al. 2001, Chiba et al. 2003). Several enzymes involved in mRNA degradation, such as poly(A) RNase, decapping enzymes and several components of the exosome, are indispensable for plant growth, suggesting that control of mRNA stability is involved in many aspects of plant development (Chekanova et al. 2000, Chiba et al. 2004, Xu et al. 2006, Iwasaki et al. 2007). Moreover, identification of cold-inducible microRNAshas suggested the involvement of mRNA stability control in the response to cold stress (Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004, Sunkar et al. 2007, Liu et al. 2008, Zhou et al. 2008, An and Chan 2012).

The half-lives of mRNA can be monitored on a genome-wide scale by conducting a set of microarray experiments over a time course following the inhibition of transcription. This strategy, called the 'mRNA decay array' in this study, has been employed to determine global mRNA half-lives in intact Arabidopsis plants or suspension cell cultures grown under normal conditions (Gutierrez et al. 2002, Narsai et al. 2007). Thus, to demonstrate the involvement of mRNA stability control in the cold stress response, we investigated global changes in mRNA half-lives in response to cold stress by mRNA decay array using an Arabidopsis suspension cell culture, T87 (Axelos et al. 1992). Single cells are accepted as a versatile model system to study the fundamental response due to the lack of complex development and organ communication (Narsai et al. 2007, Sasaki et al. 2008). Transcriptome analysis of cold stress in T87 cells has suggested that single cells share general signaling and response pathways for cold acclimation with Arabidopsis plants, although some of the cold-responsive genes were found only in cultured cells (Sasaki et al. 2008).

The present study showed that average transcripts are stabilized at low temperatures. Thus, we identified transcripts with significantly longer or shorter mRNA half-lives than those expected from the shift observed in the average transcripts as relatively stabilized or relatively destabilized, respectively. Furthermore, by conducting a functional classification, we provide insight into the functional significance of mRNA decay in response to cold stress.

Results

Changes in mRNA stability in response to cold stress

The decay rates of mRNA are typically measured by monitoring the reduction of transcript accumulation over time after transcription has been inhibited. To identify and characterize transcripts whose stability changes in response to cold stress,

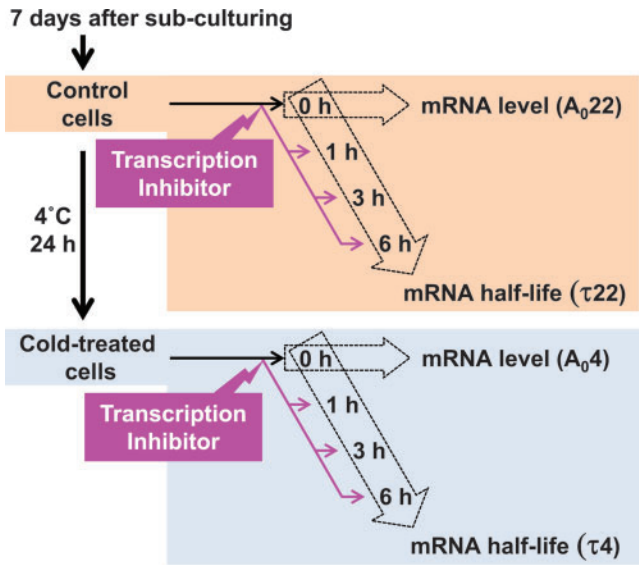

Fig. 1 Schematic representation of the mRNA decay array. Control cells were cultured for 7 d at 22°C, and then cold-treated cells were exposed to 4°C for 24 h. After adding the transcriptional inhibitor, cordycepin, total RNA was isolated at the indicated time points for microarray analysis. The experiments were performed in triplicate for each time point. mRNA half-lives in the control (τ22) and cold-treated cells (τ4) were estimated from the changes in mRNA levels following transcriptional arrest (0, 1, 3 and 6 h). mRNA accumulation levels in the control ($A_0$22) and cold-treated cells ($A_0$4) were determined by the value at the first time point (0 h), which was just before the addition of cordycepin.

mRNA half-life measurement using the transcription inhibitor cordycepin (Johnson et al. 2000), and global gene expression analysis with microarray were combined as outlined in **Fig. 1**.

T87 cells 7 d after subculturing were used as a control for the mRNA decay array, because cold acclimation was only observed in the cells during the lag phase (**Supplementary Fig. S1**; Sasaki et al. 2008). To reveal the effect of mRNA stability control on gene expression, the cells were exposed to 4°C for 24 h. This treatment condition is generally used for cold acclimation, and the largest number of genes have been shown to change their mRNA accumulation level at this time point in several transcriptome studies in Arabidopsis (Fowler and Thomashow 2002, Hannah et al. 2005, Vogel et al. 2005, Oono et al. 2006, Kilian et al. 2007).

Cordycepin effectively inhibited transcription in T87 cells (**Supplementary Fig. S2**). Total RNA was extracted from time-course samples after the addition of cordycepin and was used for microarray analysis (**Fig. 1**). The half-lives of individual transcripts showed a wide distribution, both in control and in cold-treated cells, and a bell-shaped distribution was obtained after log conversion. The average mRNA half-life in control cells was 1.8 h, whereas that in cold-treated cells was 22.7 h (**Fig. 2A**). Thus, cold treatment resulted in 13-fold longer half-lives, indicating that the average transcripts were stabilized in the cold-treated cells. Therefore, we identified the relatively stabilized and relatively destabilized transcripts as those whose half-lives were >1.5-fold longer or shorter than those expected from the general shift, respectively, and had a q-value <0.05 by Welch's t-test. From a total of 10,929 analyzed transcripts, 2,055 relatively stabilized transcripts and 1,969 relatively destabilized transcripts were selected (**Figs. 2B**, **3**; **Supplementary Table S1**). Note that the total RNase activity decreased 12-fold in the control cells at the low temperature (**Supplementary Fig. S3**).

Verification of mRNA decay array

To confirm the results of mRNA decay array, we used conventional quantitative reverse transcription–PCR (qRT–PCR) for time-course analysis after the addition of cordycepin to cells before and after cold treatment. Forty-four randomly selected transcripts from the relatively stabilized and relatively destabilized classes showed comparable mRNA half-life changes in response to cold treatment. The average Pearson's correlation coefficients between the results from the mRNA decay array and qRT–PCR were 0.90 and 0.84 for data before and after cold treatment, respectively, indicating reasonable global correlation (**Supplementary Fig. 4**).

Classification by functional category of relatively stabilized and relatively destabilized transcript classes

To explore the functional relevance of changes in mRNA stability, the distributions of relatively stabilized and relatively destabilized transcripts among the Functional Catalogue (FunCat; Ruepp et al. 2004) were analyzed. The relatively

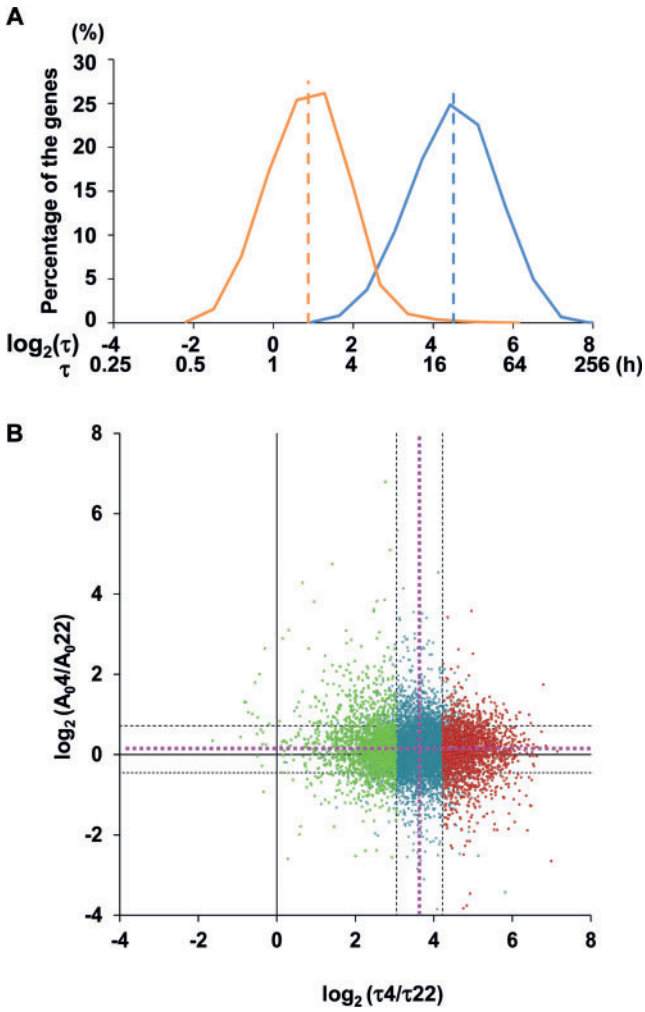

Fig. 2 Changes in mRNA half-lives and mRNA levels in response to cold stress. (A) Histograms of $\log_2$-converted mRNA half-lives before (orange line) and after (blue line) cold treatment. Dashed lines indicate averages of mRNA half-lives. (B) Scatterplot of the $\log_2$ ratio of mRNA half-lives after cold treatment to before [$\log_2(\tau 4/\tau 22)$] vs. the $\log_2$ ratio of mRNA levels after cold treatment to before [$\log_2(A_0 4/A_0 22)$]. The general shift in mRNA half-lives (vertical) and the general shift in mRNA levels (horizontal) 24 h after cold treatment are presented as magenta dotted lines. Cut-off lines at 1.5-fold difference from the general shift in mRNA half-lives (vertical) and from the general shift in 24 h mRNA levels (horizontal) are represented as gray dotted lines. Relatively stabilized and relatively destabilized transcripts are represented as red and green dots, respectively.

stabilized class was significantly enriched in the following categories: Transcription; Protein with binding function or cofactor requirement; Regulation of metabolism and protein function; Cell fate; and Cell type differentiation (**Fig. 4A**). These categories included a large number of transcription factors, RNA helicases, GTP-binding proteins, DNA- or RNA-binding proteins, kinases and phosphatases, as well as many factors related to plant growth and development (**Supplementary Tables S3–S7**). On the other hand, the relatively destabilized class was enriched in the following categories: Metabolism; Cell

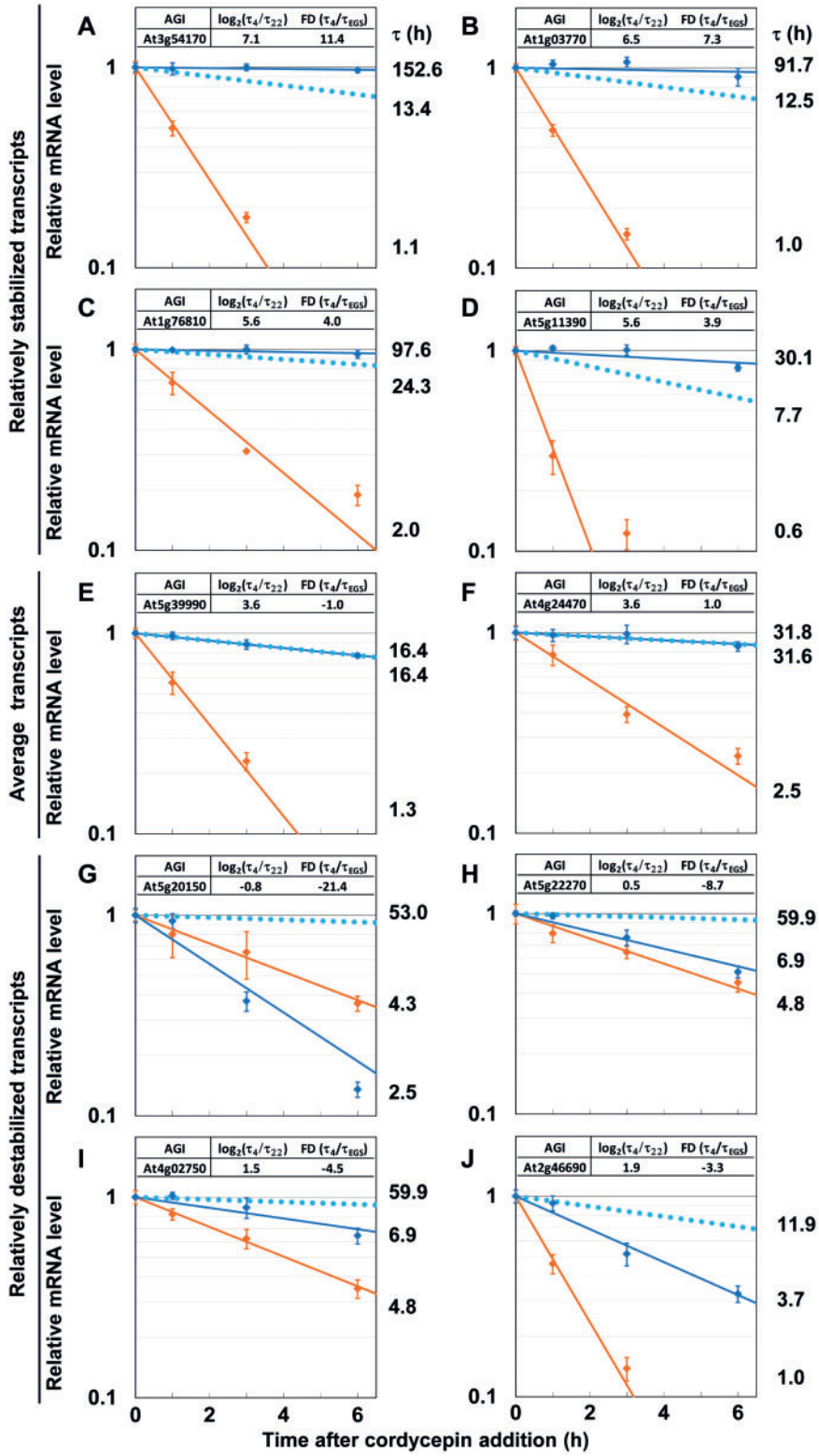

Fig. 3 mRNA half-lives determined by mRNA decay array experiments. Representative mRNA degradation patterns of relatively stabilized transcripts (A–D), average transcripts (E and F) and relatively destabilized transcripts (G–J) are shown before (orange lines) and after 24 h cold treatment (blue lines). mRNA levels at each time point were normalized to the average value at 0 h. Blue dotted lines represent mRNA degradation patterns under cold conditions that are expected from the general shift (EGS). For each transcript, Arabidopsis gene ID (AGI), $\log_2$ ratio of mRNA half-life after cold treatment to that before [$\log_2(\tau 4/\tau 22)$] and fold difference of mRNA half-lives after cold treatment to EGS [FD ($\tau 4/\tau 4_{EGS}$); values <1 are inverted and represented with a negative sign] are shown. The mRNA half-life for each line is shown on the right side of the graph. Results from three biological replicates are shown.

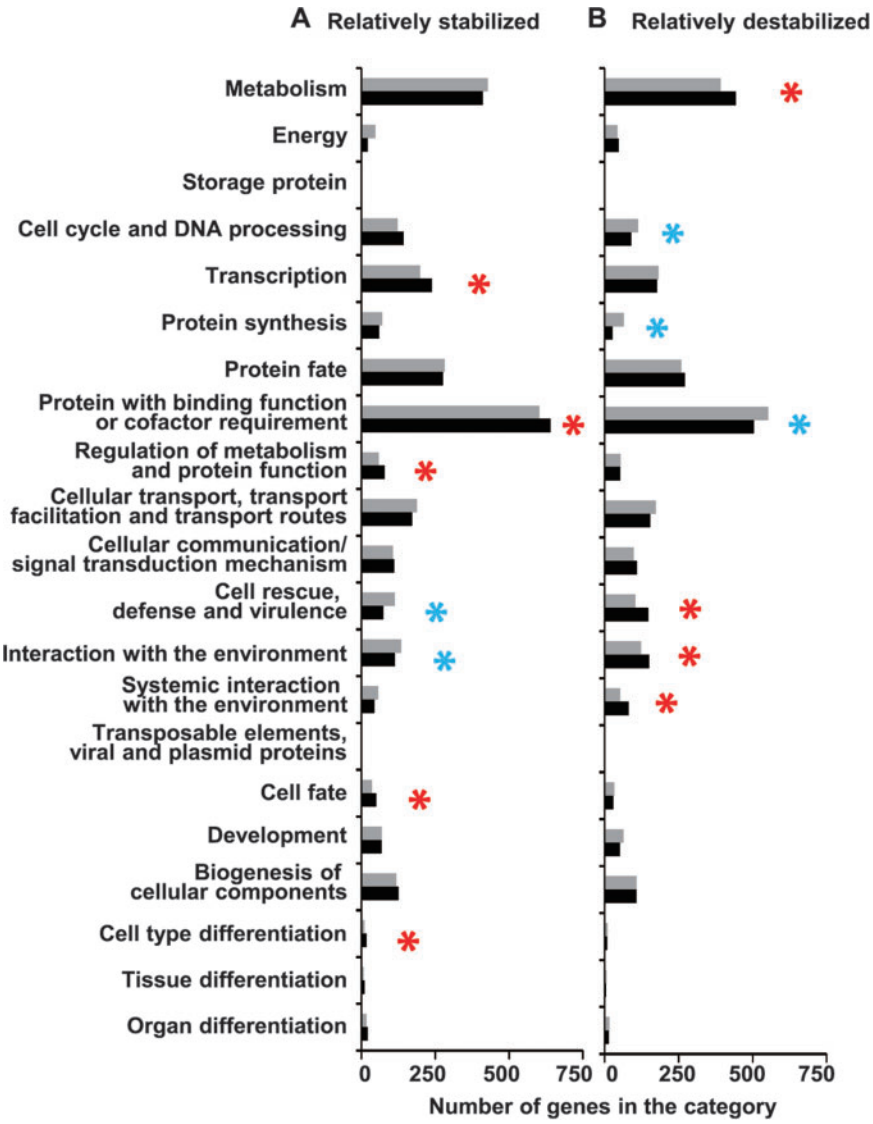

Fig. 4 Functional classification of relatively stabilized and relatively destabilized transcripts. (A) Relatively stabilized or (B) relatively destabilized transcripts in response to cold stress were classified according to the FunCat, in which 6,954 genes comprising 63.6% of the total 10,929 transcripts were annotated into 21 main categories, excluding the 'unclassified proteins' category. The expected number of genes in each category from the distribution of all analyzed transcripts is represented by gray bars, and the observed number is represented by black bars. Significantly over- or under-represented categories are marked by red or blue asterisks, respectively (two-sided Fisher's exact test, q-value <0.05).

rescue, defense and virulence; Interaction with the environment; and Systemic interaction with the environment (**Fig. 4B**). The Metabolism category included enzymes for production or degradation of various metabolites (**Supplementary Table S8**). The relatively destabilized transcripts in the Cell rescue, defense and virulence category mostly encoded stress response-related proteins and disease, virulence and defense-related proteins. Stress response-related proteins are involved in oxidative, osmotic, salt, heat, DNA damage, nutrient and radiation stresses, in addition to cold stress (**Supplementary Table S10**). The majority of relatively destabilized transcripts in the Systemic interaction with the environment category were related to plant hormonal responses, while those in the Interaction with the environment category mainly encoded proteins involved in cellular sensing and the response to external stimuli, including temperature perception and its response-related proteins (**Supplementary Tables S11, S12**).

Classification by protein domain

As another classification approach, enrichment analysis was performed using the functional annotation tools provided in DAVID (Huang et al. 2009) to classify the transcripts into functional groups based on the annotation terms of the InterPro protein families (Hunter et al. 2009). Notably, the relatively destabilized class was enriched in the pentatricopeptide repeat (PPR), cyclin-like F-box, Myb transcription factor and Myb-type helix–turn–helix (HTH) DNA-binding domain

protein families (Fig. 5; Supplementary Tables S13–S16). In particular, the PPR protein family was greatly enriched, and the number of genes in this category was 3-fold higher than expected (q-value = 9.47E-36). No enriched category was observed in the relatively stabilized class. We also performed a pathway-based classification of relatively stabilized and relatively destabilized classes using PageMan (Thimm et al. 2004). Regarding the distributions of relatively destabilized transcripts, similar results were obtained (Supplementary Fig. S5).

Correlation between mRNA half-life and mRNA accumulation level 24 h after cold treatment

mRNA levels are subject to the balance between transcription speed and degradation rate (Perez-Ortin et al. 2007). To understand the role of mRNA half-life changes in response to cold, changes in mRNA accumulation levels after cold treatment were also considered. mRNA accumulation levels were determined by microarray data before application of a transcription inhibitor (0 h, Fig. 1). Cold treatment led to a 1.1-fold increase in mRNA levels, indicating that little general shift occurs in mRNA accumulation levels 24 h after cold treatment (Fig. 2B). As in the case for mRNA half-life changes, we designated those transcripts whose accumulation levels were >1.5-fold higher or lower than those expected from the general shift and with a q-value < 0.05 by Welch's t-test as up-regulated and down-regulated transcripts, respectively. A total of 1,309 up-regulated transcripts and 1,370 down-regulated transcripts were identified (Supplementary Table S1).

The relatively stabilized and relatively destabilized transcripts were further classified according to the above criterion. The relatively stabilized transcripts were found more than expected in the down-regulated transcripts, whereas the relatively destabilized transcripts were found more than expected in the up-regulated transcripts (Fig. 6A, B). These results were unexpected because the directions of change in mRNA accumulation were opposite to those expected from the changes in mRNA half-lives. In the relatively stabilized class, down-regulated transcripts were enriched in the Cell cycle and DNA processing category (Fig. 6C; Supplementary Table S2), whereas in the relatively destabilized class, up-regulated transcripts were enriched in the Cell rescue, defense and virulence, Interaction with the environment and Systemic interaction with the environment categories (Fig. 6D).

Correlation between mRNA half-life and mRNA accumulation level at a wide temporal range of cold treatment

A cell culture system is suitable for observing short-term, but not long-term, cold responses, because obtaining a mock control is difficult due to the expeditious growth of cells under the control condition. Therefore, to consider the changes in mRNA levels over a wide temporal range of cold treatments, we referred to data from the global survey of transcriptome analyses during cold treatment in Arabidopsis plants (Hannah et al. 2005). These authors compiled published data on consistent changes for short- (<12 h), medium- (24–48 h) and long-term (48 h to 14 d) responses to cold (Fig. 7A). We subdivided the relatively stabilized or relatively destabilized transcripts based on these compiled data. The relatively destabilized class was over-represented in the long-term down-regulated transcripts and short-term up-regulated transcripts (Fig. 7C, G). FunCat analyses showed that, among the relatively destabilized class, the long-term down-regulated transcripts were enriched in the Metabolism category (Fig. 8A), whereas the short-term up-regulated transcripts were enriched in the Transcription, Cell rescue, defense and virulence, Interaction with the environment and Systemic interaction with the environment categories (Fig. 8B). Note that genes that were up-regulated at 24 h in this study were also enriched in the latter three categories (Fig. 6D). The relatively stabilized class was not enriched in any of the short-, medium- or long-term responsive genes (Fig. 7B, D, F).

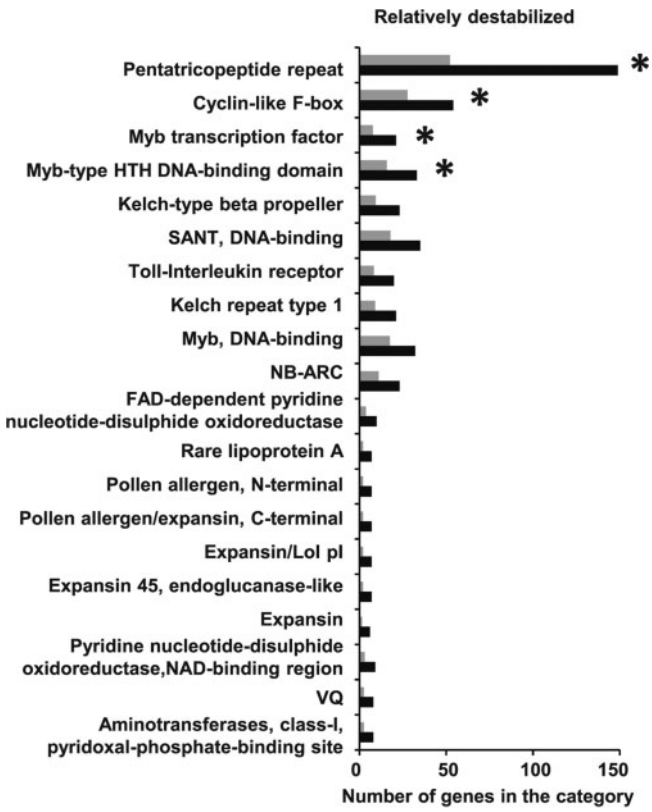

Fig. 5 Enrichment analysis with the annotation terms of the protein domains for relatively destabilized transcripts. Enrichment analysis with the annotation terms from the InterPro protein families was performed using DAVID with default settings. The expected and observed numbers of genes in each category are represented as gray and black bars, respectively. Significantly enriched annotation terms are marked with an asterisk (EASE-modified Fisher's exact test, q-value < 0.01). Overall, 149 PPR proteins, 54 cyclin-like F-box proteins, 21 Myb transcription factors and 33 Myb-type HTH DNA-binding domain proteins were identified in relatively destabilized transcripts.

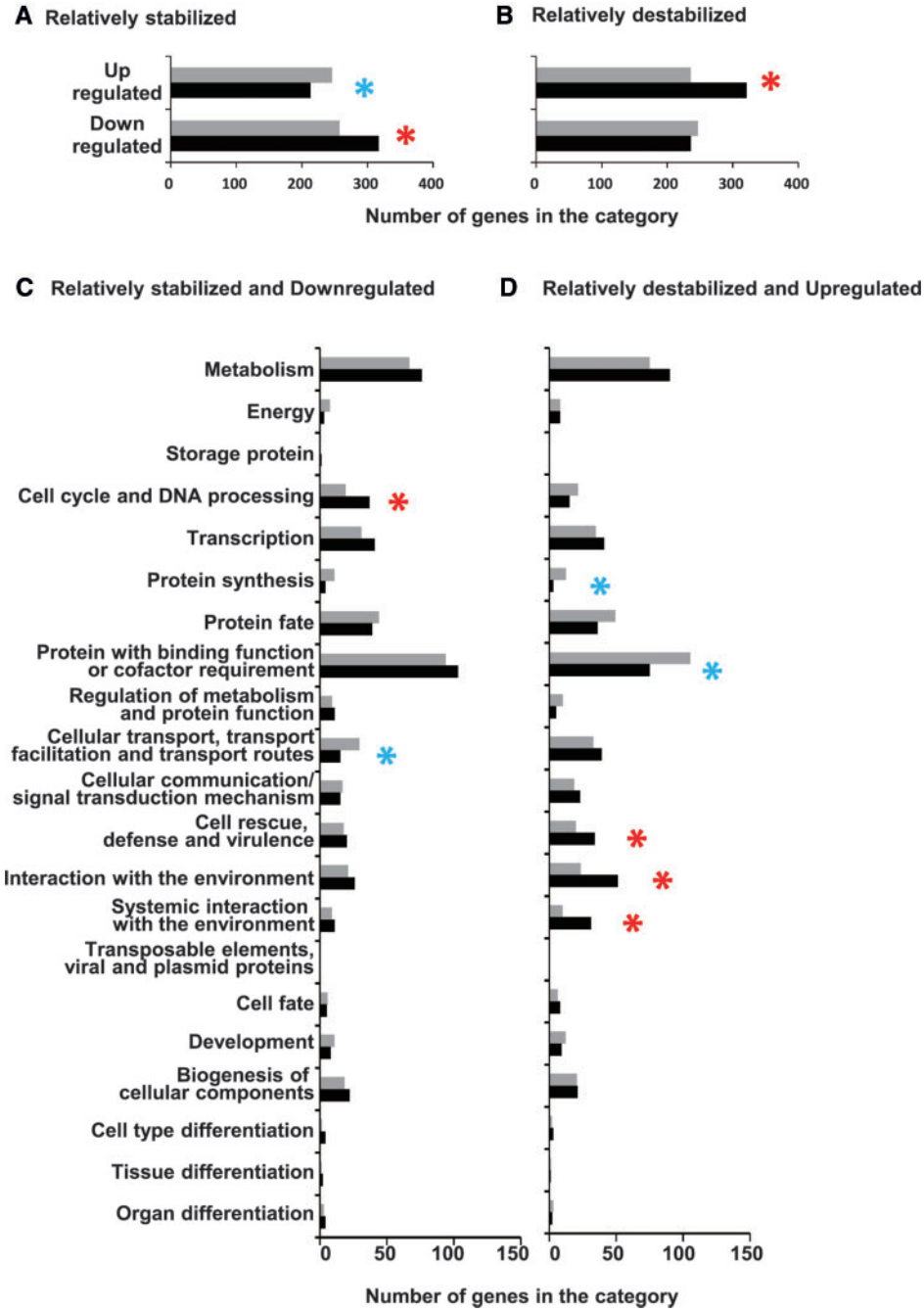

Fig. 6 Classification of relatively stabilized and relatively destabilized transcripts according to changes in mRNA levels after 24 h of cold treatment. (A) Relatively stabilized and (B) relatively destabilized transcripts were further classified according to changes in mRNA level after 24 h of cold treatment. (C) Relatively stabilized transcripts that were down-regulated at 24 h and (D) relatively destabilized transcripts that were up-regulated at 24 h were classified according to the FunCat. The expected number of genes in each category from the distribution of all analyzed transcripts is represented by gray bars, and the observed number is represented by black bars. Significantly over- or under-represented categories are marked by red or blue asterisks, respectively (two-sided Fisher's exact test, *P*-value with the Bonferroni correction <0.05).

Changes in mRNA half-life in CBF-responsive genes

CBF-responsive genes were major up-regulated transcripts in response to cold stress. To evaluate the changes in mRNA half-lives of CBF-responsive genes, we retrieved CBF-responsive genes that were up-regulated by both cold treatment and over-expression of CBFs in the published transcriptome analyses (Fowler and Thomashow 2002, Maruyama et al. 2004, Vogel et al. 2005). Among a total of 10,929 transcripts, 79 CBF-responsive genes were identified. Interestingly, CBF-responsive genes were significantly enriched in the relatively destabilized

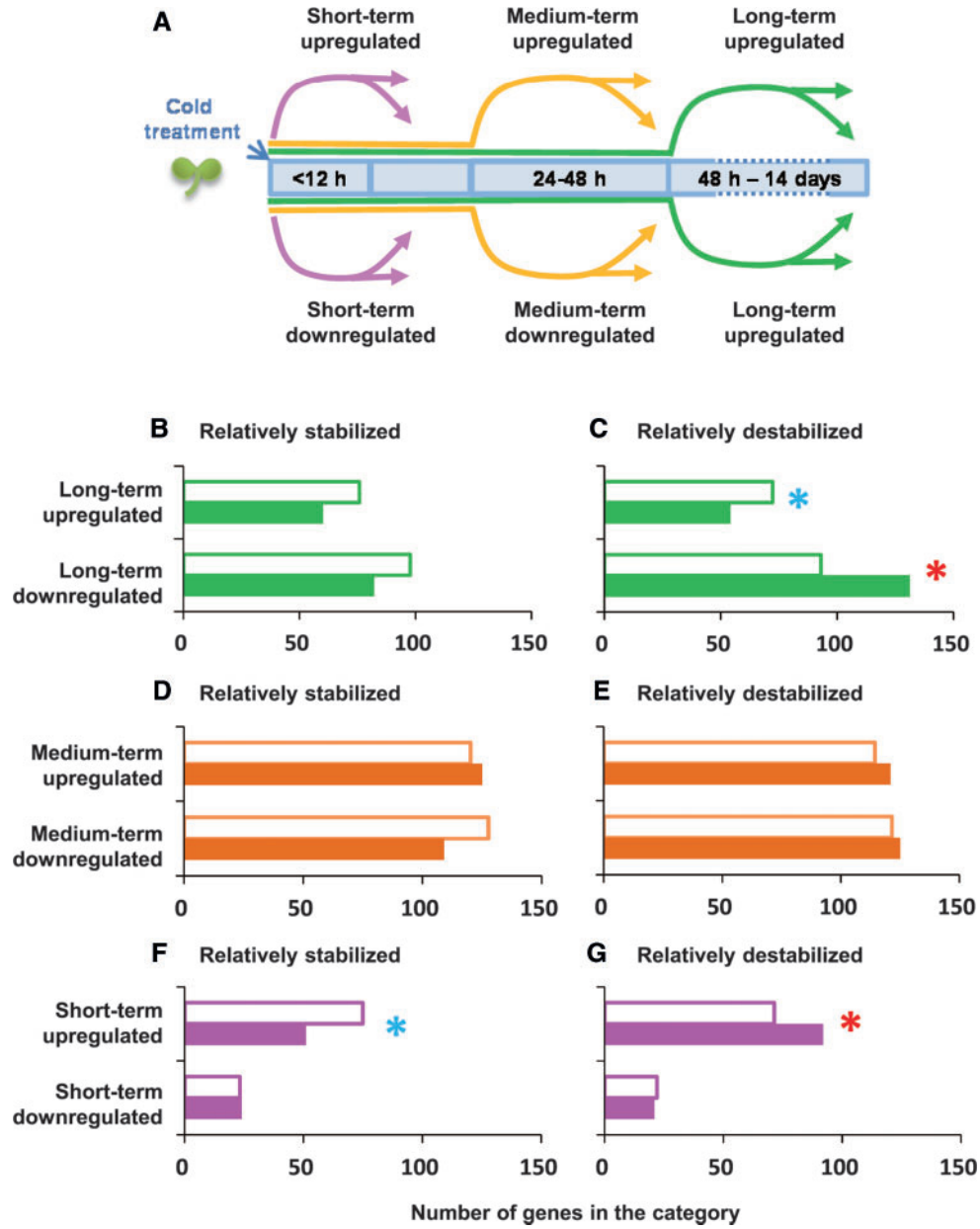

Fig. 7 Classification of the relatively stabilized and relatively destabilized transcripts according to their temporal range of cold responsiveness. (A) Schematic representation of the expression patterns with long- (green), medium- (orange) and short-term (magenta) cold-responsive genes. Relatively stabilized (B, D, F) and relatively destabilized transcripts (C, E, G) were classified according to long- (B, C), medium- (D, E) and short-term (F, G) cold responsiveness based on the compiled data of Hannah et al. (2005). The expected number of genes in each category from the distribution of all analyzed transcripts is represented by open bars and the observed number is represented by filled bars. Significantly over- or under-represented categories are marked by red or blue asterisks, respectively (two-sided Fisher's exact test, *P*-value with the Bonferroni correction <0.05).

class, although most of them were up-regulated 24 h after cold treatment (**Fig. 9**; **Supplementary Table S17**).

General structural features of genes in the relatively stabilized or relatively destabilized transcript classes

The identification of relatively stabilized and relatively destabilized transcripts allowed us to evaluate the structural properties that might be important for the control of mRNA stability under cold conditions. The distributions of specific sequence features of each class were visualized using cumulative distribution plots (**Fig. 10**) and compared with those of all analyzed transcripts. Information on the lengths of untranslated regions (UTRs) in each transcript was retrieved from the Arabidopsis Information Resource (TAIR) database version 10 (Rhee et al. 2003). When transcripts were predicted to have several splicing

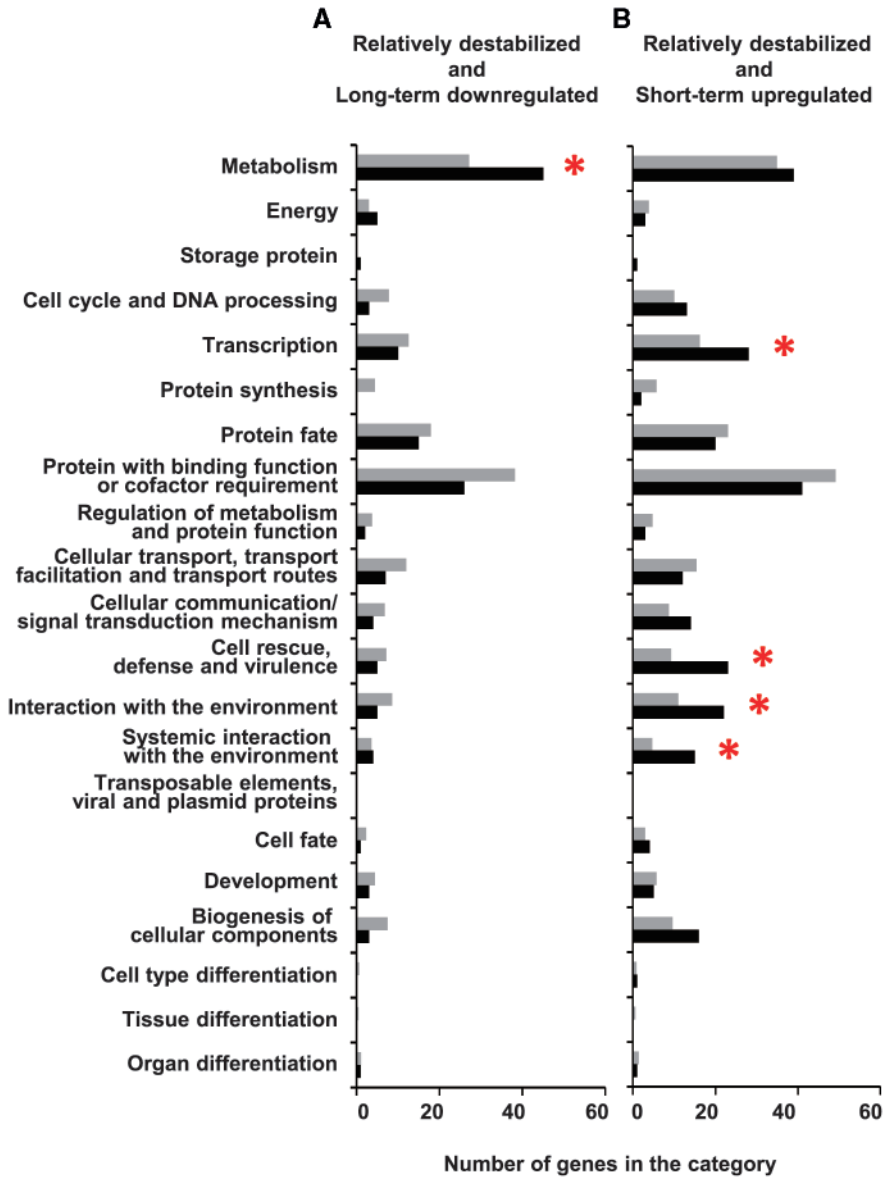

Fig. 8 Functional classification of long-term down-regulated transcripts and short-term up-regulated transcripts in the relatively destabilized transcripts. Among the relatively destabilized transcripts, long-term down-regulated transcripts (A) and short-term up-regulated transcripts (B) were classified according to the FunCat. Expected and observed numbers of genes in each category are represented by gray and black bars, respectively. Significantly over- or under-represented categories are marked by red or blue asterisks, respectively (two-sided Fisher's exact test, q-value < 0.05).

variants, information on the longest sequence in each feature was used. As shown in **Fig. 10A**, the relatively stabilized transcripts had prominently longer 5′-UTRs. In contrast, small but significant differences were observed in the 5′-UTR lengths of relatively destabilized transcripts and in the 3′-UTR lengths of relatively stabilized transcripts (**Fig. 10A, B**).

Structures in the 5′-UTR, such as upstream open reading frames (uORFs) and secondary structures, often play a major role in the regulation of translation, which is sometimes coupled with mRNA degradation (Mignone et al. 2002). Therefore, the uORF data of Arabidopsis based on the sequence information of the 5′-UTR from TAIR version 10 was used (Takahashi et al. 2012). To evaluate the presence of uORFs, the length of the longest uORF in the individual gene and all splicing variants was used. In the relatively stabilized class, the shorter uORFs consisting of <34 amino acids were significantly over-represented compared with all analyzed transcripts (**Table 1**).

The number of introns in each transcript was retrieved from the TAIR database. The relatively destabilized class was predicted to have fewer introns, whereas the relatively stabilized class was predicted to have more introns, compared with all analyzed transcripts (**Fig. 10C**). The presence of introns affects almost every step of gene expression, from transcription to translation, including mRNA stability (Le Hir et al. 2003,

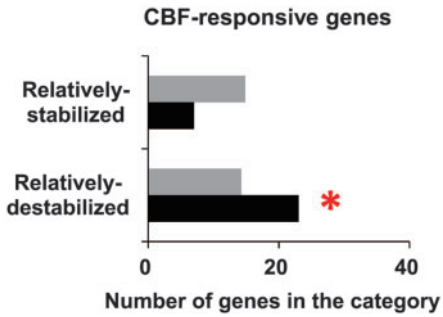

Fig. 9 Classification of CBF-responsive genes according to changes in mRNA half-life in response to cold treatment. The number of CBF-responsive genes in relatively stabilized and relatively destabilized classes is shown. Expected and observed numbers of genes in each category are represented by gray and black bars, respectively. Significantly over- or under-represented categories are marked by red or blue asterisks, respectively (two-sided Fisher's exact test, *P*-value with the Bonferroni correction < 0.05).

Wang et al. 2007). Therefore, we further classified the transcripts into three groups: 'introns-all', in which all splicing variants were predicted to have at least one intron; 'intronless-all', in which all splicing variants were predicted to have no intron; and 'intronless-some', in which one or more splicing variant was predicted to have no intron. For the relatively destabilized class, significant enrichment was observed in the intronless-all and intronless-some groups, while the relatively stabilized class was enriched in the intron-all group (**Table 2**).

To find putative *cis*-acting elements, we searched for conserved sequences in the 5′- or 3′-UTR of relatively stabilized or relatively destabilized transcripts by MEME (Bailey et al. 2006). However, only low complexity sequences with low *E*-values were found (data not shown).

Discussion

The global analysis of mRNA half-life changes in response to cold stress with mRNA decay array showed that the mRNA half-lives of average transcripts were elongated approximately 13-fold at low temperatures (**Fig. 2A**). Thus, we identified the relatively stabilized and relatively destabilized transcripts as those that had much longer or shorter mRNA half-lives, respectively, than expected from the general shift (**Fig. 2B**). Coincidence between the general shift and decrease of total RNase activity in crude extracts at low temperatures suggested that this stabilization could be due to a general decrease in enzymatic activity for mRNA degradation under low temperature conditions; however, we cannot exclude other possibilities, such as a difference in types and/or amount of RNases in the control and cold-treated cells.

Functional relevance of changes in mRNA stability in response to cold

Precise mRNA level controls are important for various aspects of cellular functions, including stress responses. mRNA levels

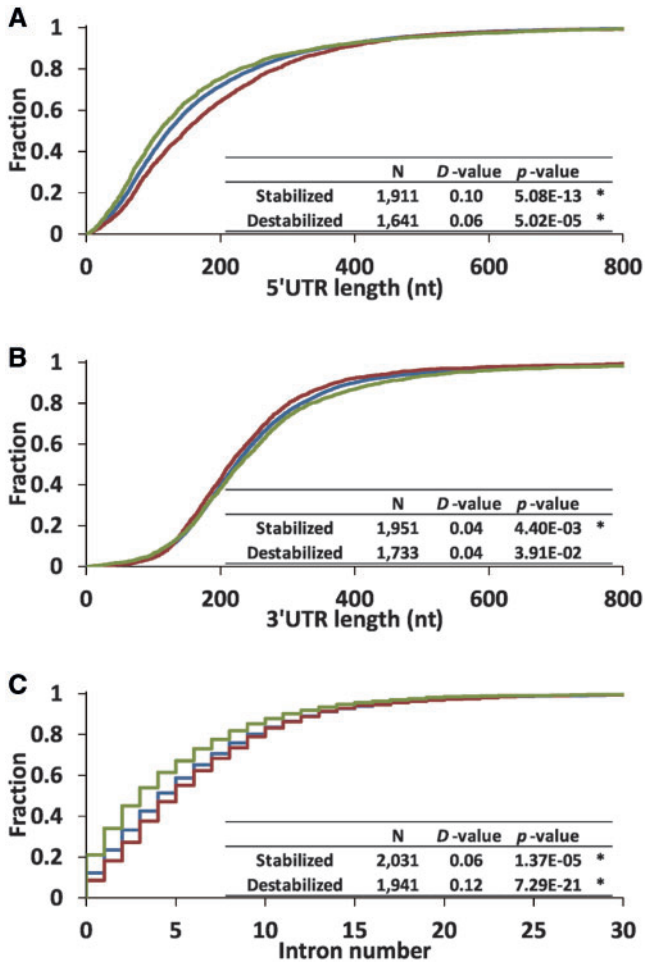

Fig. 10 Distributions of specific sequence features in each transcript class. The distributions of the length of the 5′-UTR (A), length of the 3′-UTR (B) and number of introns (C) of relatively stabilized (red line) and relatively destabilized transcripts (green line) are compared with those of all analyzed transcripts (blue line). N equals the numbers of analyzed transcripts. The *D*-value represents the distance between the distribution of each subset and that of all analyzed transcripts, and significant differences are marked with an asterisk (two-sample Kolmogorov–Smirnov test, *P*-value < 0.01).

are determined by the balance of mRNA synthesis and degradation, but the speed of transition to a new steady-state level is solely dependent on the rate of mRNA degradation (Perez-Ortin et al. 2007). For example, in the case of either an increase or a decrease in the mRNA level, the shorter half-lives enable quicker transition of the mRNA level (**Supplementary Fig. S6**). Even for transient changes in mRNA accumulation, the mRNA degradation rate is critical for determining the transition speed.

Functional classification analyses of relatively stabilized and relatively destabilized transcripts suggested reasonable functional relevance of mRNA stability controls in response to cold. In this study, we took a global view of each transcript class rather than focusing on individual transcripts. Relatively destabilized transcripts were enriched in the Cell rescue,

Table 1 Classification of relatively stabilized and relatively destabilized transcripts by the length of the longest uORF in the 5′-UTR

	All	Relatively stabilized			Relatively destabilized		
	Observed (%)	Expected	Observed (%)	*P*-value	Expected	Observed (%)	*P*-value
<34 amino acid uORF	3,073 (31.5%)	601	682 (35.7%)	2.0E-05*	516	507 (30.9%)	1.0E + 00
≥35 amino acid uORF	589 (6.0%)	115	96 (5.0%)	8.3E-02	99	115 (7.0%)	1.4E-01
No. of analyzed transcripts	9,766		1,911			1,641	

Relatively stabilized and relatively destabilized transcripts were classified by the amino acid length of the longest uORF in the individual genes including splicing variants. The observed and expected numbers of genes in each category are shown. Significant differences as compared with the distributions of all analyzed transcripts are marked with an asterisk (two-sided Fisher's exact test, *P*-value with the Bonferroni correction <0.05).

Table 2 Classification of relatively stabilized and relatively destabilized transcripts by the presence of introns

	All	Relatively stabilized			Relatively destabilized		
	Observed (%)	Expected	Observed (%)	*P*-value	Expected	Observed (%)	*P*-value
Intronless-all	1,321 (12.2%)	249	174 (8.6%)	8.37E-08*	238	410 (21.1%)	7.77E-35*
Intronless-some	156 (1.4%)	29	21 (1.0%)	2.94E-01	28	48 (2.5%)	2.05E-04*
Introns-all	9,307 (86.3%)	1753	1,836 (90.4%)	1.08E-08*	1,675	1,483 (76.4%)	2.23E-39*
No. of analyzed transcripts	10,784		2,031			1,941	

Relatively stabilized and relatively destabilized transcripts were classified into three categories; transcripts that have no intron (intronless-all), transcripts that have one or more intronless splicing variants (intronless-some) and transcripts that always contain one or more introns in their splicing variants (introns-all). The observed and expected numbers of genes in each category are shown. Significant differences as compared with the distributions of all analyzed transcripts are marked with an asterisk (two-sided Fisher's exact test, *P*-value with the Bonferroni correction <0.05).

defense and virulence, Interaction with the environment and Systemic interaction with the environment categories, suggesting that many stress and hormonal response-related genes have shorter half-lives to adjust their mRNA levels quickly to cold conditions (**Fig. 4B**). Opposing changes in mRNA accumulation and mRNA half-lives seem counterintuitive and inefficient because transcription and mRNA degradation are compensatory. However, relatively destabilized and up-regulated transcripts in early stages of the cold response were commonly enriched in these categories, also suggesting that their swift increase in mRNA levels was achieved by destabilization (**Figs. 6D**, **8B**). Moreover, CBF-responsive genes were enriched in relatively destabilized transcripts, although most are up-regulated at low temperatures (**Fig. 9**). In contrast, relatively destabilized and down-regulated transcripts in late stages of the cold response were enriched in the Metabolism category, implying that mRNA destabilization in these transcripts contributes to a decrease in their steady-state amount of mRNA at low temperatures (**Fig. 8A**).

Another point of interest was that the PPR protein family was predominantly found in the relatively destabilized class (**Fig. 5**). PPR proteins are RNA-binding proteins with essential functions in various steps of post-transcriptional regulation, such as RNA editing, splicing, stability and translation, within mitochondria and chloroplasts (Saha et al. 2007, Schmitz-Linneweber and Small 2008, Nakamura et al. 2012). Although the involvement of PPR proteins in the cold stress response has not been reported, several reports have indicated a role in the abiotic stress response through ABA signaling (Koussevitzky et al. 2007, Zsigmond et al. 2008, Liu et al. 2010, Laluk et al. 2011). Little information is available on the regulation of PPR protein genes; however, the expression of two *PPR protein-like* (*PPRL*) genes, which encode negative regulator of defense, has been reported to be down-regulated by different types of pathogen-inducible small interfering RNAs (siRNAs), probably through mRNA degradation (Katiyar-Agarwal et al. 2006, Katiyar-Agarwal et al. 2007).

The relatively stabilized transcripts were significantly over-represented in the functional categories, containing several regulators for transcriptional, post-transcriptional and post-translational controls (**Fig. 4A**). Stabilization of mRNA could be a strategy to save energy and increase the mRNA levels rather than accelerating de novo synthesis of mRNA, particularly at low temperatures. Therefore, one may find it tempting to surmise that these regulators are required for the production of specific proteins to survive in the cold, and that their mRNA half-lives are elongated to increase mRNA levels. Consistent with this view, mRNA stabilization is an important factor for controlling several cold-inducible genes in *Escherichia coli* (Gualerzi et al. 2003) and for the induction of RNA helicase expression under cold stress in cyanobacteria (Chamot and Owttrim 2000).

Sequence features of transcripts and mRNA stability control

Physical sequence analyses of relatively stabilized and relatively destabilized transcripts revealed two interesting structural features. First, relatively stabilized transcripts had much longer 5′-UTRs than other transcripts, and uORFs were significantly over-represented in these relatively stabilized transcripts (**Fig. 10A**, **Table 1**). Several lines of evidence have indicated a link between translational control and mRNA degradation; for example, translation of the uORFs can trigger nonsense-mediated mRNA decay (NMD), a eukaryotic mRNA quality

control system to identify and degrade mRNA containing premature termination codons (Lee and Schedl 2004, Mendell et al. 2004). Plant uORFs can also trigger NMD in a size-dependent manner, and uORFs with ≥35 amino acids efficiently induce NMD (Nyiko et al. 2009). Considering that shorter uORFs with <34 amino acids were significantly over-represented in relatively stabilized transcripts, NMD is unlikely to be involved in the stabilization of these transcripts in response to cold stress. However, we cannot exclude the possibility that these shorter uORFs are involved directly or indirectly in other mRNA stability controls.

Secondly, relatively destabilized transcripts were enriched in the intronless-all and intronless-some groups (**Table 2**). Accumulating data from genome sequencing and global analysis of mRNA half-lives have revealed that mRNA half-lives of intronless transcripts are much shorter than those of intron-containing transcripts in Arabidopsis and some types of human cells (Narsai et al. 2007, Wang et al. 2007). Considering this correlation, these physical sequence features may be related to the regulation of mRNA half-life in response to cold stress in some relatively destabilized transcripts. The mechanisms of how the mRNA stability is enhanced by introns and how the system is utilized for responding to cold stress remain to be elucidated. However, in the case of relatively destabilized transcripts categorized as intronless-some, alternative splicing could be one possible strategy. Recent transcriptome analysis using deep sequencing analyses has revealed that at least 42% of intron-containing genes in Arabidopsis are alternatively spliced. Moreover, various abiotic stresses, including cold stress, were associated with alternative splicing events (Filichkin et al. 2010). In particular, the intron-retaining type of alternative splicing, which can produce intronless transcripts, preferentially occurred under cold stress (Iida et al. 2004).

Conclusion

The present study demonstrates that changes in mRNA half-lives in response to cold stress are observed in a large number of transcripts. Moreover, functional classification analysis of relatively stabilized and relatively destabilized transcripts provides an overview of their possible biological roles related to changes in mRNA stability, suggesting that control of mRNA stability could be a versatile strategy for regulating gene expression in response to cold stress.

Materials and Methods

Arabidopsis T87 cell culture and cold treatment conditions

Arabidopsis T87 suspension cells derived from Columbia ecotype were grown in JPL media (Axelos et al. 1992) at 22°C under constant light (80 μmol m^{-2} s^{-1}) with orbital shaking (120 r.p.m.). Cells were transferred to new medium every 2 weeks at ~20-fold dilution. Seven days after subculturing, cells were divided into two batches. One batch was used as control cells, and the other was transferred to 4°C under dim light (40 μmol m^{-2} s^{-1}) and cultured for 24 h.

Transcription measurement by [^{3}H]uridine incorporation

Various concentrations of codycepin were added to the T87 cell cultures and incubated with gentle shaking for 30 min. Subsequently, cells were treated with 370 kBq ml^{-1} [^{3}H]uridine (1.11 TBq $mmol^{-1}$) for 2 h and then collected by a gentle centrifugation. Total RNA was extracted from the cells with the RNeasy Plant Mini kit (Qiagen), and poly(A) RNA was then selected from an equal amount of total RNA using the GenElute mRNA Miniprep kit (Sigma). The incorporation of [^{3}H]uridine into poly(A) RNA was measured using a liquid scintillation counter after trichloroacetic acid precipitation.

RNase activity assay

Preparation of crude protein extracts from cell samples was carried out as described in Yen and Green (1991) with some modifications. We used a neutral extraction buffer (150 mM citrate-$NaHPO_4$ buffer, pH 5.6, 0.1 mM phenylmethylsulfonyl fluoride) for homogenization. To prepare ^{3}H-labeled poly(A) RNA substrates, control cells were treated with 370 kBq ml^{-1} [^{3}H]uridine (1.11 TBq $mmol^{-1}$) for 4 h and then collected. Total RNA extraction and poly(A) RNA preparation were performed as described in the previous section. The same amounts of ^{3}H-labeled mRNA substrate were incubated at 22 or 4°C with several dilution series of crude protein extracts. After incubation for 0, 10, 30 and 60 min, the remaining level of the ^{3}H-labeled poly(A) RNA substrate was measured as described in the previous section. The mRNA degradation rate was calculated from changes in the ^{3}H count relative to that at 0 min. The mRNA degradation rate per μg of protein was designated as total RNase activity.

Data processing of mRNA decay array and half-life calculation

To achieve >90% transcription inhibition, 200 and 300 μg ml^{-1} cordycepin was added to control and cold-treated cells, respectively. Cells were collected prior to cordycepin treatment (0 h), and 1, 3 and 6 h after the start of cordycepin treatment. Total RNA was extracted from the cells using the RNeasy Plant Mini kit, and purified and concentrated with the RNeasy MinElute Cleanup kit (Qiagen). Biotin-labeled cRNA was prepared from equal amounts of total RNA using one-cycle target labeling, and hybridized to Affymetrix GeneChip Arabidopsis ATH1 genomic arrays. All procedures were carried out following the Affymetrix GeneChip Expression Analysis technical manual. Bioconductor software was used to analyze the microarray data (Gentleman et al. 2004). To extract the expression level of the probe set on each chip, the Robust Multichip Average (RMA) method (Irizarry et al. 2003) was used. The

log-transformed RMA value was delogged and then normalized with the average expression level of poly(A) RNA controls derived from *Bacillus subtilis* genes. Although the RMA method was used for extraction of expression levels from raw data in this study, the *P*-value for present and absent calls of the 'mas5calls' function (Liu et al. 2002) in Bioconductor (Hubbell et al. 2002) is useful to find probe sets with ambiguous signals (McClintick and Edenberg 2006, Mieczkowski et al. 2010). Therefore, we excluded transcripts from the following analysis if their *P*-values were higher than 0.06 in at least two out of three replicates from the 0 h data point. Additional ambiguous probe sets, marked as possible cross-hybridizations, and probes derived from organelle genomes, were removed. Based on the above filtering process, a total of 13,373 transcripts were used for the subsequent analyses.

To estimate the decay constant (λ) for each transcript, the change in the mRNA level (A) relative to the average value at 0 h was fitted to an exponential regression model without Y-intersection, $A = \exp(-\lambda t)$, where t is time after the start of cordycepin treatment (hours). The λ and its SE (SEλ) were estimated by non-linear regression using the least-squares method (Narsai et al. 2007). The decay constant of individual transcripts in control cells was estimated after omitting the 6 h time point if a smaller SEλ was obtained. The mRNA half-life (τ) was then calculated from the equation, $\tau = \ln(2)/\lambda$. The value of A at $t = 0$ (A_0) was obtained by averaging the normalized microarray signal values at $t = 0$ of three replicates. To evaluate the quality of data fitting to the exponential model described above, residual SD of fitting (SD_r) was calculated. Microarray data with either $SD_r/A_0 \geq 0.2$, $SE\lambda/\lambda \geq 1$ or $\lambda \leq 0$ were eliminated from further analyses, resulting in a final microarray data set of 10,929 transcripts. For statistical analyses, the q-value was calculated using the false discovery rate (Benjamini and Hochberg 1995) to adjust multiple comparisons.

Classification and characterization of relatively stabilized and relatively destabilized transcripts

Functional classification of relatively stabilized and relatively destabilized transcripts was carried out based on the FunCat assigned by the Munich Information Center for Protein Sequences (http://mips.helmholtz-muenchen.de/proj/funcatDB/). For classification by protein domains, we used the functional annotation tool provided by DAVID (http://david.abcc.ncifcrf.gov/summary.jsp) with default settings. A pathway-based classification was performed by PageMan downloaded from http://mapman.gabipd.org/web/guest/mapman. The putative conserved motifs in the UTRs were searched using the MEME suite (http://meme.sdsc.edu/meme/intro.html).

qRT–PCR analysis

Total RNA was extracted from frozen cell samples using the RNeasy Plant Mini kit and then converted to cDNA, followed by DNase I treatment using the protocol provided with SuperScript II (Invitrogen). qRT–PCR was performed using SYBR premix ExTaq (TAKARA) under conditions presented in the protocol attached for the Mx3005P instrument (Stratagene). Primers used are listed in **Supplementary Table S18**.

Evaluation of freezing tolerance

Freezing tolerance was evaluated by measuring electrolyte leakage from cells, as described in Inada et al. (2006), with some modifications. Briefly, cells were washed with 0.08 M sorbitol three times and suspended in the same solution to 4 mg μl^{-1}. Cells were divided into test tubes containing 20 µl each, and set in a programmed freezer pre-cooled to 0°C. After immediate cooling to −1°C, we followed the EQF procedure (Inada et al. 2006). The test tubes were thawed to 4°C overnight in the dark, and then 1 ml of 0.08 M sorbitol was added to each sample. After gentle agitation at room temperature for 1 h in the dark, ion leakage was measured with a B-173 conductivity meter (Horiba). Relative survival rate was calculated by normalizing the values of ion leakage from an unfrozen sample to 0% and a lethal frozen sample to 100%.

Supplementary data

Supplementary data are available at PCP online.

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