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Arabidopsis HDA6 is required for freezing tolerance

Taiko Kim To [a,b], Kentaro Nakaminami [a], Jong-Myong Kim [a], Taeko Morosawa [a], Junko Ishida [a], Maho Tanaka [a], Shigeyuki Yokoyama [b], Kazuo Shinozaki [c], Motoaki Seki [a,d,*]

[a] Plant Genomic Network Research Team, RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[b] Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Gene Discovery Research Group, RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[d] Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama, Kanagawa 244-0813, Japan

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ABSTRACT

Many plants exhibit altered gene expression patterns in response to low nonfreezing temperatures and an increase in freezing tolerance in a phenomenon known as cold acclimation. Here we show, for the first time, that the histone deacetylase gene *HDA6* is required for cold acclimation and freezing tolerance in *Arabidopsis*. *HDA6* is transcriptionally upregulated during long-term cold treatment. Cold-treated *hda6* mutants showed reduced freezing tolerance compared with the cold-treated wild-type plants. Freezing-caused electrolyte leakage increased in the cold-treated *hda6* mutant. In contrast, the non-cold-treated *hda6* mutants showed no significant difference in survivability and electrolyte leakage compared to wild-type plants. Transcriptome analysis identified the genes that showed aberrant expression in the *hda6* mutant after cold acclimation. We conclude that *HDA6* plays a critical role in regulating cold acclimation process that confers freezing resistance on *Arabidopsis*.

1. Introduction

Low temperature is one of the major environmental stresses that causes agricultural yield losses and limits the geographic regions where crops can be grown [1]. Many plants, including *Arabidopsis*, have the ability to increase their freezing tolerance through a process called cold acclimation by which gene expression is altered in response to low nonfreezing temperatures [1–4]. Research over the decades has shown that many physiological and molecular changes occur during cold acclimation. Three DREB/CBF-type transcription factors, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2, are known to be involved in the cold response [4–6]. These transcription factors are rapidly induced by cold [7–9] and regulate the induction of many *RD*/*COR* (responsive to desiccation/cold-regulated) genes. Overexpression of DREB1B/CBF1 or DREB1A/CBF3 in *Arabidopsis* constitutively activates a series of downstream genes and enhances freezing tolerance [5,10–15]. Inducer of CBF expression 1 (ICE1) positively regulates the upregulation of DREB1A/CBF3 expression in a sumoylation-dependent manner [16–18]. A majority of the most highly expressed cold standard (COS) genes were induced in response to DREB1C/CBF2 expression, however, over 70% of the cold-induced COS genes and 95% of the cold-repressed COS genes were not identified as its regulon [19], and a novel mechanism, independent of these transcription factors, has been suggested.

The involvement of chromatin regulation in cold response has been pointed out and several genes in chromatin regulation, for example the bromodomain proteins, were reported to be cold regulated [17]. ADA2B, a transcriptional co-activator, has been shown to interact physically with a histone acetyltransferase GCN5 and DREB1B/CBF1 [20–22]. In the *ada2b-1* mutant, the expression levels of several COR genes were downregulated but freezing tolerance was enhanced without cold treatment, suggesting the involvement of *ADA2B* in repressing a freezing tolerance pathway in warm temperature [21]. HOS15, a WD40-repeat protein identified in a screen of altered abiotic stress signaling, was suggested to control cold regulated genes through histone deacetylation [23,24]. The *hos15* mutant was hypersensitive to freezing temperatures and the level of acetylated histone H4 was higher in the *hos15* mutant than in wild-type plants [24]. Although the importance of histone modification in gene regulation is well known in eukaryotes, the direct evidence for the contribution of histone modifying enzymes in freezing tolerance has not been reported. This study is the first to define the role of *Arabidopsis* histone deacetylase *HDA6* in cold acclimation and freezing tolerance.

* Corresponding author at: Plant Genomic Network Research Team, Plant Functional Genomics Research Group, RIKEN Plant Science Center (PSC), RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan. Fax: +81 45 503 9584.
E-mail address: mseki@psc.riken.jp (M. Seki).

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2. Materials and methods

2.1. Plants and growth conditions

All experiments used the *Arabidopsis hda6* mutants, *axe1–5* and its parental line DR5 (ecotype Columbia) [25] and *sil1* [26,27] and its wild-type plants (ecotype Landsberg *erecta*). Unless otherwise stated, growth conditions were as follows: seeds were surface-sterilized and stratified for 4 days at 4 °C in the dark; the seeds were then sown and grown in tissue culture plates on MS agar (0.8%) medium supplemented with 1% sucrose under 16 h light/8 h dark for 14 days at 22 °C.

2.2. Freezing tolerance assay

The *hda6* mutants and wild-type plants were grown on the same culture plates for 2 weeks. The plates were then incubated for 3 h at −2 °C and a freezing nucleus was created by freezing the plate edge quickly in liquid nitrogen. For non-cold-treated plants, the temperature was decreased to −12 °C with a 1 °C decrease every 2 h in the dark. For the cold acclimation test, 3 days of cold treatment at 2 °C (12 h light/12 h dark) was done prior to the freezing treatment during which the temperature was decreased to −18 °C with a 1 °C decrease every hour in the dark. Frozen plates were thawed slowly in the dark at 4 °C for 12 h and then grown at 22 °C for 10 days under 16 h light/8 h dark conditions. The numbers of plants that survived were counted and the mean and standard deviation was calculated.

2.3. Electrolyte leakage assay

The fully developed rosette leaves of soil-grown 3–4 weeks old plants were used to determine freezing-caused electrolyte leakage as described earlier [18] with modifications. Four excised leaves (two each of the third and fourth true leaves) were placed in a glass tube containing 10 ml of deionized water and the tube was set in a low-temperature thermostat bath (RTE-7, Thermo Fisher Scientific) filled with ethanol. The tubes were incubated at −2 °C for an hour and liquid nitrogen-frozen zirconia beads were added to the tubes to create freezing nuclei. The temperature was then set to decrease to −10 °C with a 0.5 °C decrease every 15 min. Tubes were taken from the bath at intervals of −2 °C and thawed at 4 °C overnight in the dark prior to evaluation of injury. The conductivity was measured with a conductivity meter (B-157, Horiba). The total conductivity for each sample was determined after autoclaving at 121 °C for 20 min. Percent electrolyte leakage averaged over 4–5 replicates was plotted for each freezing temperature.

2.4. RNA preparation

Total RNA was extracted from the plants either before or after cold treatment (at 2 °C for 3 days) using Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions.

2.5. RT-PCR

cDNA was synthesized with QuantiTect reverse transcription kit (Qiagen). RT-PCR analysis was performed using the Ex Taq DNA polymerase kit (Takara) and 1–4 ng of cDNA. The PCR conditions were as follows: pre-incubation for 5 min at 94 °C; 27–32 cycles at 94 °C for 20 s, 58 °C for 20 s, 72 °C for 1 min; and a final extension at 72 °C for 4 min. Primers are listed in Supplemental Table 1.

2.6. Microarray analysis

Microarray analysis was performed using the total RNA extracted from 2 weeks old plants with and without 3 days of cold treatment at 2 °C. The cDNA was synthesized using 500 ng of total RNA and labeled with one color (Cy3) using Quick Amp labeling kit (Agilent Technologies), followed by fragmentation and hybridization to the *Arabidopsis* oligo DNA microarray Ver. 4.0 (Agilent Technologies). Three biological replicates were performed for each genotype. The microarray data are available on the GEO website (GEO ID: GSE26873).

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. Genes with at least a 2-fold difference in their expression levels were evaluated with the Student's *t*-test and genes with *p*-values < 0.05 were counted as differentially expressed in the mutant.

3. Results

3.1. The upregulation of HDA6 under low temperature treatment

The expression analysis revealed that the histone deacetylase gene *HDA6* was transcriptionally upregulated by long-term cold treatment (Fig. 1). In contrast to the rapid upregulation of *DREB1B/CBF1*, *DREB1A/CBF3* and *RD29A/COR78/LTI78*, the expression level of *HDA6* was upregulated only slightly in the first 24 h and strongly upregulated after 3 days (72 h) of cold treatment. This result indicates the involvement of *HDA6* in the cold response in *Arabidopsis*.

3.2. Cold-treated hda6 mutants exhibit reduced freezing tolerance

Because *HDA6* is transcriptionally upregulated by low temperature (Fig. 1), we compared the freezing tolerance of the loss of function *hda6* mutant *axe1–5* [25] and its wild-type plants DR5. When the plants were exposed to freezing stress in the absence of cold treatment, little difference was observed in the survival rates of the wild-type and mutant plants and, after freezing stress at −12 °C, only 20% of the wild-type and mutant plants survived (Fig. 2A).

The cold acclimation process increased the plants viability after freezing stress. Most of the cold-treated DR5 plants acquired the ability to survive at −18 °C (Fig. 2B). Notably, the cold-treated *axe1–5* plants exhibited significantly reduced survivability to freezing stress compared to the cold-treated DR5 (Fig. 2B and C). The survival rates of the *axe1–5* plants started to decrease at −16 °C and only one third of the mutant plants survived at −18 °C. The survival rates at −18 °C of the mutant plants were approximately 3-folds less than that of DR5. The other *hda6* mu-

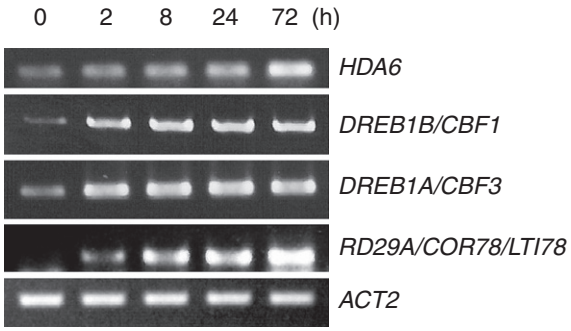

Fig. 1. Expression patterns of *HDA6* and several cold responsive genes examined by RT-PCR. Plants were incubated for 0, 2, 8, 24, 72 h at 2 °C.

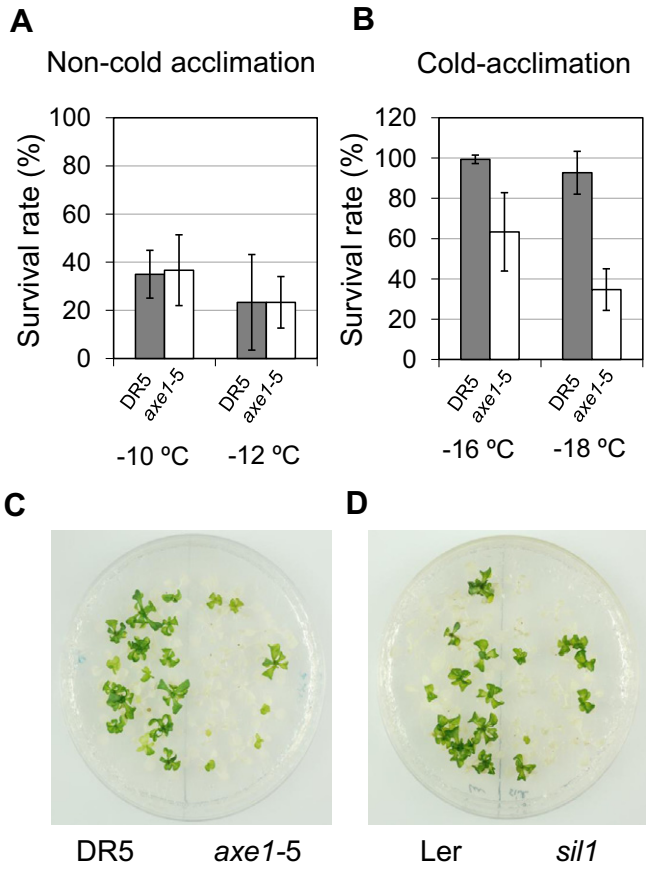

Fig. 2. Freezing tolerance of wild-type and *hda6* mutant plants after freezing stress. (A) The survival rates of non–cold-treated or (B) cold-treated plants after freezing stress at the temperatures indicated. The values are the means and standard deviations of the results from 10 plates (15 plants per plate) of DR5 and the *hda6* mutant *axe1–5* (total $n = 150$). (C and D) Photographs of wild-type and *hda6* mutant plants 10 days after freezing treatment at −18 °C. (C) Wild-type DR5 and the *hda6* mutant *axe1–5*. (D) Wild-type Landsberg *erecta* and the *hda6* mutant *sil1*.

tant *sil1* [26,27] also showed decreased freezing tolerance (Fig. 2D, Supplemental Fig. 1). These results strongly suggest that *HDA6* is required for the cold acclimation process by which the plants acquire freezing tolerance.

3.3. Cold-treated axe1–5 mutants show increased freezing-caused electrolyte leakage

The involvement of *HDA6* in freezing tolerance and in cold acclimation capacity was also assessed by measuring freezing-caused electrolyte leakage [18]. Fully developed rosette leaves of 3–4 weeks old plants before and after pre-incubation under cold (at 4 °C for 3 days) were excised and examined. The leaves of non-cold–treated plants displayed high levels of electrolyte leakage in both the wild-type and mutant plants with no significant difference between the two (Fig. 3A). When the plants were cold-treated, the electrolyte leakage caused by the freezing injury was much more in the *axe1–5* mutant than in the cold-treated DR5 plants at all the temperatures tested in this study (Fig. 3B). These results indicate that the *axe1–5* mutant failed to fully acclimate to low temperature, resulting in the more severe damage observed in the mutant after freezing stress.

3.4. Expression patterns of cold responsive genes

Plants that exhibit altered freezing tolerance often also display aberrant expression of cold responsive genes [4,6]. To investigate if

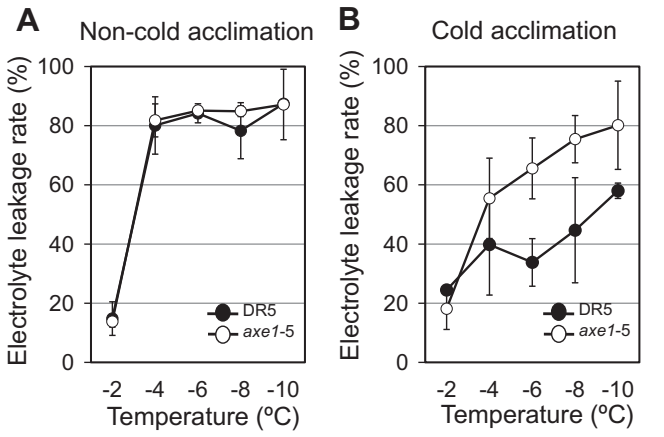

Fig. 3. Electrolyte leakage assay to measure freezing-caused electrolyte leakage from the leaves of cold-treated (3 days at 4 °C) and non–cold-treated plants. The values are the means of 4–6 replicates with standard deviation. (A) Non-cold-treated DR5(●) and *axe1–5* mutant (○). (B) Cold-treated DR5 (●) and *axe1–5* mutant (○).

the decreased freezing tolerance of the *hda6* mutants was caused by aberrant expression of the genes involved in cold responses, we examined the expression patterns of some transcription factors such as *DREB1A/CBF3*, *DREB1B/CBF1*, and *MYB15,* and other cold responsive genes such as *RD29A/COR78*, *RD29B/LTI65*, and *RD17/COR47* under cold conditions. We found that all these genes were induced in both the *axe1–5* mutant and in DR5 and no significant differences in the regulation of these genes were observed (Supplemental Fig. 2).

3.5. Microarray analysis under cold acclimation

To identify the genes differently expressed in the *axe1–5* mutant after cold acclimation, we performed microarray analysis and compared the expression profiles of DR5 and the *axe1–5* mutant plants after cold treatment. Based on the criteria ($\geqslant$2-fold difference in expression, Student's *t*-test $p < 0.05$), 517 genes were identified as being affected by the *axe1–5* mutation after cold acclimation. Of the affected genes, 482 genes were upregulated and 35 genes were downregulated in the *axe1–5* mutant (Fig. 4A, Supplemental Tables 2 and 3). The genes were classified into three categories according to their responsiveness in the DR5 plants; cold-induced, cold-repressed, or with little significant difference (Fig. 4A). Of the upregulated genes in the *axe1–5* mutant, 108 genes were classified as cold-repressed and 37 genes as cold-induced in the wild-type plants. Of the downregulated genes in the mutant, 13 genes were classified as cold-induced and 7 genes as cold-repressed in the wild-type plants (Fig. 4A). The differential regulation of several representative genes in the *axe1–5* mutant was confirmed by semi quantitative RT-PCR (Fig. 4B and C).

The genes that exhibited increased expression in the *axe1–5* mutant represent a wide range of biological functions (Fig. 4B): transcription factors (*At5g05660* ATNFXL2, *At3g53310*); cellular metabolism (*At3g30720* QQS, *At1g47840* HXK3); pathogen defense (*At2g17430* MLO7); and ion homeostasis (*At2g32830* PHT5). Several genes, such as protein chaperon BiP3 (*At1g09080*) and protein secretion ATSAR1 (*At1g09180*) which are involved in the ER stress response, displayed abnormal upregulation in response to low temperatures only in the *axe1–5* mutant and not in DR5.

The downregulated genes in the *axe1–5* mutant include genes with several functions (Fig. 4C). Notably, several cold inducible genes showed lower induction levels in the *axe1–5* mutant compared to in DR5, including genes involved in lipid composition change such as fatty acid desaturase (*At1g06100*) and LTP3

(*At5g59320*). A histone demethylase gene *IBM1* was also found to be upregulated by cold treatment in DR5 and less induced in the *axe1–5* mutant. The genes involved in senescence (*At5g13170* SAG29), chlorophyll biosynthesis (*At5g54190* PORA), transcriptional regulation (*At4g32280* IAA29, *At2g40435*), and another lipid transfer protein (*At4g33550*) were all downregulated in the *axe1–5* mutant.

Our data show that the aberrant regulation of gene expression occurred after cold acclimation in the *axe1–5* mutant, suggesting that the proper regulation of these genes is important for cold acclimation and freezing tolerance of plants.

4. Discussion

The physiological and transcriptome analyses presented in this study show that the loss of function mutants of *HDA6* display reduced freezing tolerance and altered gene expression after cold acclimation, suggesting that the *HDA6* gene has a critical role in cold acclimation and freezing tolerance. The comprehensive transcriptome analysis under low temperature identified 517 genes with altered expression in the *hda6* mutant (Fig. 4A, Supplemental Tables 2 and 3). The DREB/CBFs mediated cold response pathways were not significantly affected by the *axe1–5* mutation, suggesting the existence of a novel cold responsive pathway mediated by *HDA6*. This is the first evidence that a mutation in a histone modifying enzyme can cause altered freezing tolerance without changing the expressions of the well characterized cold responsive genes. The genes with aberrant responses in the *hda6* mutant might include new candidates for the acquisition of freezing tolerance.

Both the *axe1–5* and *sil1* mutants showed reduced freezing tolerance only after cold treatment (Fig. 2). In addition, *HDA6* transcription was revealed to be upregulated by cold (Fig. 1). Thus, we propose that *HDA6* functions in regulating the cold acclimation process under low temperature condition. The observation that a longer period of cold treatment increases plants freezing tolerance [3] suggests that *Arabidopsis* plants needs longer period to respond further and accomplish cold acclimation. As the induction of *HDA6* is a relatively late response to cold (Fig. 1), the function of HDA6 in the cold response might be manifested in a later step. Our data suggest that the cold acclimation process includes an early response regulated by DREB/CBFs and a later response that involves chromatin-level regulation mediated by *HDA6*.

If the differentially expressed genes in the *axe1–5* mutant at normal temperature (22 °C) cause the freezing sensitivity of the *hda6* mutants, then the freezing tolerance without cold acclimation should also be altered. However, both the *axe1–5* and *sil1* mutants show reduced freezing tolerance only when cold–treated prior to freezing, suggesting that the genes with altered expression especially after cold treatment have a role in freezing tolerance. In the *axe1–5* mutant, several genes show weaker responses in their induction/repression and several genes show abnormal responses that were not seen in the wild-type plants (Fig. 4B and C).

The importance of genes that influence lipid composition and cellular membrane fluidity has been suggested in freezing tolerance. There are some evidences that the overexpression of fatty acid desaturase improves freezing tolerance in several plant species [28–31]. The overexpression of the *Arabidopsis* lipid transfer protein EARLY1 was also reported to reduce electrolyte leakage caused by freezing damage [32]. A member of the plant lipid trans-

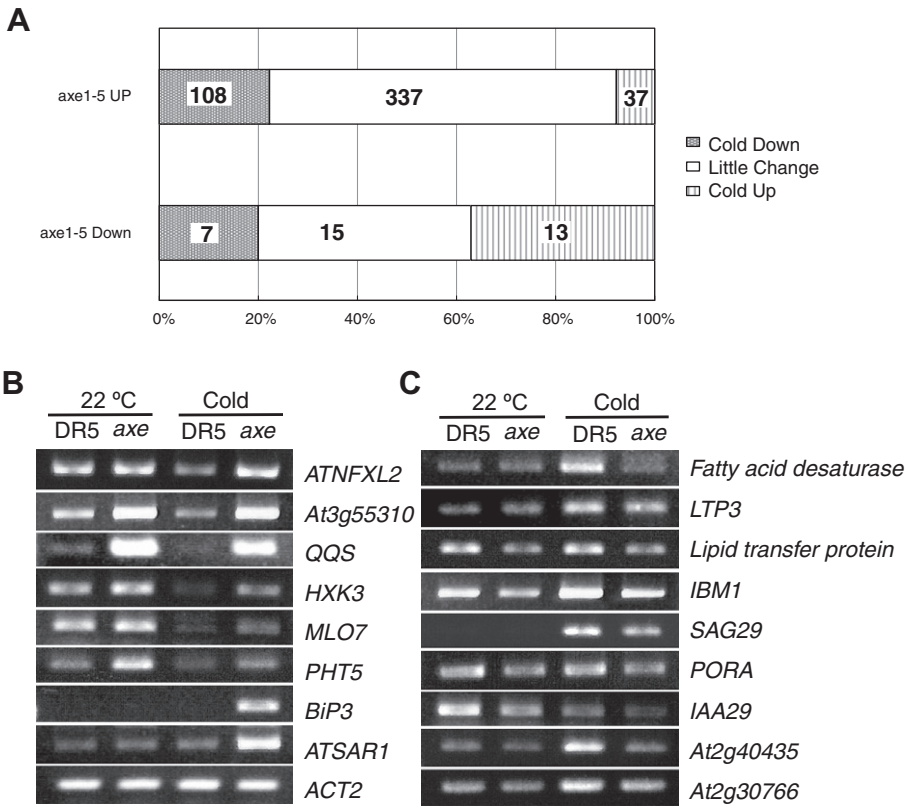

Fig. 4. Transcriptome analyses of DR5 and the *axe1–5* mutant after cold treatment. (A) The classification of differentially expressed genes in the *axe1–5* mutant after cold treatment according to their cold responsiveness in the DR5 plants. (B and C) Expression patterns of differentially expressed genes in the *axe1–5* mutant identified in microarray analysis were validated by RT-PCR. *ACT2* were served as a control. (B) Upregulated genes in the *axe1–5* mutant after cold treatment. (C) Downregulated genes in the *axe1–5* mutant after cold treatment.

fer protein family, cryoprotectin, has cryoprotective activity in cabbage [33]. Interestingly, some of these genes, the fatty acid desaturase gene *At1g06100* and the lipid transfer protein genes *LTP3* and *At4g33550*, show lower expression in the *hda6* mutant under cold conditions (Supplemental Table 3, Fig. 4C). Thus, we suggest that the downregulation of these genes in the *hda6* mutant may be one of the causes for the lower freezing tolerance of the *hda6* mutant.

Interestingly, the microarray analysis in this study revealed that many of the other histone deacetylases were also upregulated by cold. The upregulation of the histone deacetylases, *HDA9, HDA19, SRT2*, *HD2A*, *HD2B* and *HD2C*, was validated by RT-PCR analysis (Supplemental Fig. 3). Most of these genes show gradual and relatively late induction similar to what was observed for *HDA6* (Fig. 1). These results suggest that *Arabidopsis* responds to long-term cold stimulus by upregulating *HDA6* and other histone deacetylases to induce alterations in chromatin status and structure that confers freezing tolerance on the plants.

The mutants of histone acetyltransferase *gcn5–1* and transcriptional co-activator *ada2b-1* showed altered inducibility of *COR* genes [21], suggesting that histone modification may affect cold signaling. It is interesting that not only *HDA6* but several other histone deacetylases are induced by cold stimulus (Fig. 1, Supplemental Fig. 3). These histone deacetylases may function either in parallel or redundantly with *HDA6* in the cold acclimation process to acquire freezing tolerance. The importance of histone deacetylation during cold acclimation is supported by the report that the *hos15* mutant with increased histone acetylation levels showed reduced freezing tolerance [24]. Several genes with different expression in the *hos15* mutant [24] are also differentially expressed in the *axe1–5* mutant, indicating the possibility that *HOS15* and *HDA6* may function in overlapping pathways. Further analysis of the role of histone modification in cold acclimation will reveal new insights into plant cold responses and contribute to improvements in the freezing tolerance of crop plants.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.058.

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