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RDM4 modulates cold stress resistance in *Arabidopsis* partially through the *CBF*-mediated pathway

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Summary

- The *C-REPEAT-BINDING FACTOR* (*CBF*) pathway has important roles in plant responses to cold stress. How the *CBF* genes themselves are activated after cold acclimation remains poorly understood.
- In this study, we characterized cold tolerance of null mutant of *RNA-DIRECTED DNA METHYLATION 4* (*RDM4*), which encodes a protein that associates with RNA polymerases Pol V and Pol II, and is required for RNA-directed DNA methylation (RdDM) in *Arabidopsis*.

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Author contributions

Z.C. and J-K.Z. planned and designed the research; Z.C., Y.W., M.C., Y.G., Z.M., H.W., Y.H., X.D. and X-J.H. performed experiments; Z.C. and Y.W. analyzed data; Z.C. and J-K.Z. wrote the manuscript.

Supporting Information

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- The results showed that dysfunction of *RDM4* reduced cold tolerance, as evidenced by decreased survival and increased electrolyte leakage. Mutation of *RDM4* resulted in extensive transcriptomic reprogramming. *CBFs* and *CBF* regulon genes were down-regulated in *rdm4* but not *nrpe1* (the largest subunit of PolV) mutants, suggesting that the role of *RDM4* in cold stress responses is independent of the RdDM pathway. Overexpression of *RDM4* constitutively increased the expression of *CBFs* and regulon genes and decreased cold-induced membrane injury. A great proportion of genes affected by *rdm4* overlapped with those affected by *CBFs*. Chromatin immunoprecipitation results suggested that RDM4 is important for Pol II occupancy at the promoters of *CBF2* and *CBF3*.
- We present evidence of a considerable role for *RDM4* in regulating gene expression at low temperature, including the *CBF* pathway in *Arabidopsis*.

Keywords

C-REPEAT-BINDING FACTOR (CBF); cold; Pol II; RNA-directed DNA methylation; *RNA-DIRECTED DNA METHYLATION 4 (RDM4)*; transcriptome

Introduction

As sessile organisms, plants are frequently exposed to a wide range of adverse environmental conditions. Among such stress conditions, cold temperatures greatly limit plant growth and agricultural productivity. To survive freezing conditions, plants increase their freezing resistance when exposed to low, nonfreezing temperatures in a process called cold acclimation (Thomashow, 1999; Stockinger *et al.*, 2001; Shi *et al.*, 2014). Cold acclimation involves adjustment of metabolism and growth and extensive changes in gene expression in plants (Kreps *et al.*, 2002; Fowler *et al.*, 2005; Hannah *et al.*, 2005). Analyses of cold-regulated genes led to the discovery of the *C-REPEAT BINDING FACTOR* (*CBF*) pathway for cold-responsive gene expression. *Arabidopsis* encodes three cold-inducible *CBF* genes, that is, *CBF1*, *CBF2*, and *CBF3* (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999; Maruyama *et al.*, 2004; Vogel *et al.*, 2005; Yamaguchi-Shinozaki & Shinozaki, 2006). Constitutive or stress-inducible overexpression of *CBF1*, *CBF2*, and *CBF3* in transgenic plants resulted in enhanced expression of *COLD-REGULATED* (*COR*) genes and increased freezing resistance in *Arabidopsis* (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2004).

Compared with the considerable information available concerning *CBF* downstream gene expression changes, less is known about how the *CBF* genes themselves are activated after cold acclimation. To date, two positive regulators of *CBFs* have been identified: *INDUCER OF CBF EXPRESSION 1* (*ICE1*), a Myc family transcription factor (Chinnusamy *et al.*, 2003; Lee *et al.*, 2005), and *CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3* (*CAMTA3*), a CAMTA family transcription factor (Doherty *et al.*, 2009). Another factor that affects the expression of *CBF1*, *CBF2*, and *CBF3* is the circadian clock (Bieniawska *et al.*, 2008; Kidokoro *et al.*, 2009; Dong *et al.*, 2011). Two transcription factors that are core components of the clock, that is, *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) and

LATE ELONGATED HYPOCOTYL (*LHY*), were recently determined to regulate the *CBF* cold-response pathway.

In eukaryotes, the task of transcribing nuclear genes is shared between three RNA polymerases: RNA polymerase (Pol) I, Pol II and Pol III (Schramm & Hernandez, 2002; Woychik & Hampsey, 2002; Grummt, 2003). In addition, plants have evolved two additional DNA-dependent RNA polymerases, known as Pol IV and Pol V, that specifically transcribe genomic regions that must be silenced epigenetically through the RNA-directed DNA methylation pathway (Pikaard *et al.*, 2008; Ream *et al.*, 2009). In the last two decades, numerous transcriptional regulators have been found to regulate transcriptional activity of Pol II (Egloff & Murphy, 2008; Fuda *et al.*, 2009). It is unclear to what extent these transcriptional regulators may also be involved in Pol IV and Pol V function.

In a previous study (He *et al.*, 2009), we identified a transcriptional regulator, *RNA-DIRECTED DNA METHYLATION 4* (*RDM4*), that is required for RNA-directed DNA methylation (RdDM) in *Arabidopsis*; *RDM4* was also known as *DEFECTIVE IN MERISTEM SILENCING 4* (*DMS4*) (Kanno *et al.*, 2010). The *rdm4*/*dms4* mutants exhibited pleiotropic developmental phenotypes and showed changes in the expression of protein-coding genes. The RDM4 protein is associated with Pol IV and Pol V as well as with Pol II (He *et al.*, 2009; Kanno *et al.*, 2010), which is similar to its orthlogs in yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, and humans (Pagé *et al.*, 2003; Aouida *et al.*, 2004; Krogan *et al.*, 2006; Peiró-Chova & Estruch, 2009; Czeko *et al.*, 2011; Esberg *et al.*, 2011). Microarray analysis showed that the expression of several cold stress-responsive genes is reduced in the *rdm4* mutant compared with the wild-type (WT) under normal conditions (He *et al.*, 2009). These observations showed that *RDM4* is a transcriptional regulator that is shared among Pol II, Pol IV and Pol V, and suggested that *RDM4* may play a role in plant resistance to cold stress.

In the present study, we assayed cold stress resistance of *rdm4*/*dms4* mutants and *RDM4* overexpression lines. The results indicated that *rdm4/dms4* is hypersensitive to both chilling and freezing stresses. Both *rdm4* and *dms4* mutants accumulated more hydrogen peroxide (H_2O_2) and decreased amounts of antioxidant enzymes before and after cold stress treatment than the WT. Mutation of *rdm4/dms4* resulted in extensive transcriptomic changes, including down-regulation of *CBF*s and *CBF* regulon genes under both nonstress and cold stress conditions. By contrast, dysfunction of *NRPE1*, the largest subunit of Pol V, caused impaired RdDM (He *et al.*, 2009) but did not affect the expression of cold-responsive genes. We found that overexpression of *RDM4* resulted in increased cold resistance and up-regulation of *CBF*s and *CBF* regulon genes. Chromatin immunoprecipitation (ChIP) PCR assay indicated that Pol II occupancy at *CBF2* and *CBF3* promoters is lower in *rdm4* mutant plants compared with the WT under cold stress. These results suggest that *RDM4* is important for Pol II transcription of *CBFs* and other cold stress-responsive genes and is thus critical for cold stress resistance in *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

Yellow fluorescent protein (YFP)-tagged RDM4 transgenic *Arabidopsis thaliana* L. Heynh. plants driven by the CaMV 35S promoter and *rdm4* and *nrpe1* mutants in a C24 background was screened in our previous study (He *et al.*, 2009). The *dms4-1* mutant in Columbia (Col) background was kindly provided by Prof. Marjori Matzke (Kanno *et al.*, 2010) and 35S::*RDM4* overexpression lines were created in this study. All seeds were imbibed at 4°C for 4 d in the dark to promote the uniformity of seed germination. Plants were grown under a 16: 8 h, 23: 18°C, light: dark cycle in a growth room at 150 μmol m^{-2} s^{-1} and 70% relative humidity.

Cold treatment and electrolyte leakage test

For a chilling sensitivity test, seeds were placed on plates containing 0.8% MS medium and grown at 22°C after 2 d of stratification at 4°C. Chilling was initiated immediately after radicle emergence by incubating the seedlings at 4°C with 30 μmol m^{-2} s^{-1} of continuous light. Survival rates were calculated after 4months of chilling treatment. In another test, 2-wk-old plants grown in soil were treated at 4°C in a cold room before they were subjected to an ion leakage test, which was conducted essentially as described by Dong *et al.* (2009). In brief, leaf samples were detached and transferred to tubes with 10ml of deionized water. The conductivity of the solution was measured after shaking overnight at room temperature. The samples were then autoclaved for 30min. The conductivity of the solution was measured again after shaking at room temperature for 6 h. The percentage of electrolyte leakage was calculated as the percentage of the conductivity before autoclaving relative to the conductivity after autoclaving.

A freezing resistance assay was performed as previously described (Lee *et al.*, 2005). In brief, seeds were stratified at 4°C for 2 d and germinated on plates containing 0.8% MS medium at 22°C under 30 μmol m^{-2} s^{-1} continuous light. Ten-day-old seedlings were cold-acclimated at 4°C in the light (30 μmol m^{-2} s^{-1}) for 4 d. The plates with plants were placed on ice in a freezing chamber (Percival Scientific, Perry, IA, USA) at −1°C for 16 h. Ice chips were sprinkled on these plants before the chamber was programmed to cool at 1°C h^{-1}. After being frozen at desired temperatures for 2 h, the plates with plants were kept at 4°C for 12 h in the dark and were then transferred to a growth chamber at 22°C under 30 μmol m^{-2} s^{-1} continuous light. Survival of the seedlings was scored visually after 2 d. In a separate experiment concerning electrolyte leakage caused by freezing, fully developed rosette leaves were treated as previously described (Ishitani *et al.*, 1998). In brief, one excised leaflet from 3-wk-old plants was placed in a test tube containing 100 μl of deionized H_2O, and the tube was placed in a refrigerated circulator (freezing bath) (VWR Scientific, San Francisco, CA, USA) at 0°C. The temperature of the bath was programmed to decrease to −12°C in steps of 1°C every 30 min. When the designated temperature was reached (−2, −4, −6, −8, −1 or −12°C), replicate tubes were removed from the bath and placed immediately on ice to allow gradual thawing. The leaflets then were carefully transferred to another tube containing 25 ml of deionized water and shaken overnight, and the conductivity of the solution was measured before and after autoclave. The percentage of electrolyte leakage was calculated as

described earlier. Each treatment was represented by five biological replicates, and the whole experiment was repeated three times.

Determination of H_2O_2 concentrations and antioxidant enzyme activities

H_2O_2 contents were determined as described previously (Wang *et al.*, 2013). In brief, total protein was extracted with PBS buffer (50 mM, pH 7.8) from cold-treated and nontreated plants. For quantification of H_2O_2, the absorbance of the supernatant was measured at 405 nm. The H_2O_2 concentration was calculated according to a standard curve generated with known concentrations of H_2O_2. Activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were measured as previously described (Wang *et al.*, 2013). One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of WST-1 by 50%. One unit of CAT activity was defined as the amount of enzyme required to catalyze 1 μM H_2O_2 in 1 min at 25°C. The activities were expressed as fold-changes relative to the WT (Col) under control conditions.

Gene expression analysis

For Affymetrix (Santa Clara, CA, USA) GeneChip array analysis, total RNA from *rdm4* and C24 WT seedlings with or without cold treatment (0, 3 or 48 h) was extracted with the RNeasy Plant Mini Kit (Qiagen) and used to make biotin-labeled cRNA targets. Fragmented cRNAs (15 μg) were hybridized to the 24K GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix) according to the manufacturer's instructions. The arrays were scanned with a GeneChip Scanner 3000 (Affymetrix), and raw image files were converted to probe set data (*.CEL files), using the Affymetrix GeneChip Operating Software according to the manufacturer's instructions. All of the raw data (*.CEL files) were then analyzed using the affylmGUI package (Wettenhall *et al.*, 2006) in the statistical computing environment R (Gentleman *et al.*, 2004). For Agilent Oligo Microarray analysis, total RNA was extracted and purified from 35S::*RDM4* and Col WT seedlings as described earlier. A 150 ng quantity of total RNA was used to prepare a cyanine- 3 (Cy3)-labeled probe with the help of Low RNA Input Linear Amplification/Labeling kits (Agilent Technologies, Santa Clara, CA, USA), and 1.65 μg of labeled cRNA probes was fragmented using fragmentation buffer and hybridized to the *Arabidopsis* arrays according to the manufacturer's instructions. Array images were acquired with Agilent's dual-laser microarray scanner (Agilent Technologies). GeneSpring software (Agilent Technologies) was used to calculate intensity ratios and fold-changes. Two biological replicates from different growth chambers were prepared for each combination of ecotype and cold treatment. The normalized microarray data were submitted to the Gene Expression Omnibus (GEO) database with accession numbers GSE63184, GSE63185 and GSE63186. All genes with a *P*-value ≤ 0.05 and a fold-change ≥ 2 were chosen for further analysis and are listed in Supporting Information Tables S1 and S2.

Gene ontology term enrichment analysis

All genes with *P*-values ≤ 0.05 and fold-change ≥ 2.0 were loaded and annotated in GOeast (http://omicslab.genetics.ac.cn/GOEAST/index.php) (Zheng & Wang, 2008). The log odds ratio was calculated as follows: sample frequency of each category in each comparison/ background frequency of each category in the whole experiment.

Cluster analysis

The data sets of specific genes were imported into the Cluster program (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/) (de Hoon *et al.*, 2004). Hierarchical cluster analysis was performed using an uncentered matrix and complete linkage method. Resulting tree figures were prepared using the software package Java Treeview (http://jtreeview.sourceforge.net/).

ChIP assay

Chromatin immunoprecipitation was performed as described by Bowler *et al.* (2004). In brief, leaves were collected from 14-d-old C24 WT and *rdm4* mutant plants. Fresh leaf samples (2 g per plant) were immediately immersed in 1% formaldehyde for crosslinking the DNA with DNA-binding proteins. The chromatin pellets were then isolated and sheared by sonication. Pol II antibody (Santa Cruz Biotech, Dallas, TX, USA) and green fluorescent protein (GFP) antibody (Invitrogen, Carlsbad, CA, USA) were used to immunoprecipitate the DNA-Pol II and DNA-RDM4 complexes, respectively. The DNA was eluted with proteinase K and purified with phenol/chloroform/isoamyl alcohol. Real-time PCR was performed to determine the enrichment of DNA fragments with the specific primers listed in Table S3.

Statistical analysis

All experiments in this study were repeated at least three times, and the results are shown as means ± SE. Samples of every experiment were harvested from at least 30 seedlings per genotype. Spss 13.0 software (Chicago, IL, USA) was used for statistical analysis. A one-way analysis of variance (ANOVA) followed by *t*-test was used to determine whether effects of treatments were statistically different at $P < 0.05$.

Results

The *rdm4* mutant is more sensitive to chilling and freezing stresses

The *rdm4* mutant exhibits delayed seed germination and shows pleiotropic developmental phenotypes when grown in soil (He *et al.*, 2009). To minimize the effect of delayed development, we plated *rdm4* mutant seeds on the nutrient agar medium 1 wk earlier than C24 WT seeds. At 4 wk after germination at room temperature, growth did not differ between *rdm4* and C24 seedlings (Fig. 1a, upper row). After 4 months at 4°C, however, survival was < 20% for *rdm4* seedlings but was almost 100% for C24 WT seedlings (Fig. 1a middle row,b). When seedlings were exposed to freezing stress, survival was also lower for *rdm4* seedlings than for C24 WT seedlings (Fig. 1a, lower row,c).

Although rdm4 showed delayed development, no stressed phenotype was observed in *rdm4* and WT at room temperature (Fig. 2a,b). In soil, leaves of *rdm4* plants turned transparent and white (Fig. 2c) while leaves of C24 WT plants remained healthy (Fig. 2d) after 14 d at 4°C. Consistent with higher transparent leaf symptom of *rdm4* plants (Fig. 2e), significantly higher electrolyte leakage (EL) was observed on *rdm4* plants than on C24 plants (Fig. 2f). These results indicated that *rdm4* plant is sensitive to both chilling and freezing stress treatments.

Chilling and freezing stresses cause greater membrane injury in the *rdm4* mutant than in the wild-type

Chilling or freezing stress changes the structure of the cell membrane, making it leaky and resulting in the loss of ions that are vital for proper cell functioning (Uemura *et al.*, 1995). The degree of membrane injury in plants exposed to low temperatures can be measured by increases in conductivity resulting from the leakage of electrolytes from tissues. Throughout the period of 4°C treatment, the *rdm4* mutant exhibited significantly higher EL than the C24 WT. At 16 wk after chilling treatment, the EL value was 50% for the *rdm4* mutant but only 26% for the C24 WT (Fig. 3a). When seedlings were subjected to freezing without cold acclimation, EL values did not differ between *rdm4* and the C24 WT (Fig. 3b). When seedlings were subjected to freezing following cold acclimation, EL values were lower for the C24 WT than for *rdm4* (Fig. 3c), indicating mutation of *rdm4* resulted in increased membrane injury.

Overexpression of *RDM4* reduces cold-induced membrane injury

RDM4 overexpression lines were then created and verified by both northern blot analysis and microarray data from this study (Fig. 1a,b). When seedlings were exposed to chilling for up to 16 wk, EL values were lower in *RDM4* overexpression lines than in the Col WT (Fig. 3d). EL values did not differ between *RDM4* overexpression lines and the Col WT after freezing treatment without cold acclimation (Fig. 3e) but were lower in *RDM4* overexpression lines than in the Col WT after freezing treatment with cold acclimation (Fig. 3f).

RDM4/*DMS4* but not *NRPE1* mediates resistance to chilling in *Arabidopsis*

After determining that the mutation of *RDM4* in the C24 ecotype resulted in a cold-hypersensitive phenotype, we then asked whether the *dms4-1* mutant allele in the Col ecotype had the same phenotype. The results indicated that the *dms4* mutant also exhibited increased chilling sensitivity, as evidenced by the appearance of the seedlings and by EL values (Fig. 4a,b). To determine whether the role of *RDM4* in chilling resistance may be associated with its function in the RNA-directed DNA methylation pathway, we tested the *nrpe1* mutant with chilling, as NRPE1 is the largest subunit of RNA polymerase V and plays key roles in the RdDM pathway (He *et al.*, 2009). After long-term chilling, *nrpe1* and the Col WT did not significantly differ in appearance or EL value (Fig. 4a,b). We then measured the changes in expression level of *CBF* and *CBF* regulon genes in these three genotypes after 0, 3, and 48 h at 4°C. As expected, the expression levels were highest for *CBF1-3* after 3 h of chilling, while expression levels of *COR15a*, *COR15b* and *RD29a* were highest after 48 h of chilling (Fig. 4c–h). Notably, expression levels of *CBF* genes at 3 h and *CBF* regulon genes at 48 h after chilling treatment in *dms4* mutant were significantly lower than those of WT. Although *nrpe1* mutant showed increased *CBF1* expression and decreased *COR15b* expression relative to WT, no significant differences were observed between *nrpe1* mutant and WT for other tested genes (Fig. 4c–h). These results indicated that *RDM4*/*DMS4*, not *NRPE1*, might mediate plant chilling resistance.

Transcriptome analysis indicates enriched gene ontology terms including cold in the *rdm4* mutant

Global transcriptome analysis was performed to characterize the effects of the *rdm4* mutation and *RDM4* overexpression on gene expression level. As expected, expression of *RDM4* (At2g30280) was significantly decreased in the *rdm4* mutant but increased in the *RDM4* overexpression lines (Fig. S1), and chilling significantly increased expression levels of *CBF*s and *CBF* target genes in WT plants, which was consistent with reports by Gilmour *et al.* (1998, 2004) (see later Fig. S3c,d).

In general, 463, 612 and 721 transcripts were regulated after mutation of rdm4 before and 3 and 48 h after the chilling treatment, respectively (Fig. S2a). However, overexpression of the *RDM4* gene caused expression level changes of only 198–279 transcripts (Fig. S2c). Chilling treatment resulted in extensive transcriptome reprogramming in all genotypes except *RDM4* overexpressor at 3 h after the treatment (Fig. S2b–d).

Gene ontology (GO) term enrichment analysis was carried out to characterize *RDM4*- and chilling-affected pathways in *Arabidopsis*. The results indicated that GO terms related to lipid, reactive oxygen species (ROS), nitrogen, secondary metabolites, etc. were greatly overrepresented in *rdm4* mutant plants subjected or not subjected to chilling and in WT plants subjected to chilling (Table 1). Interestingly, response to cold (GO:0009409) and response to temperature stimulus (GO:0009266) were enriched after chilling treatment and in rdm4 mutant. These results showed that modulation of *RDM4* changed downstream gene expressions, including cold stress-responsive genes.

Mutation of *RDM4*/*DMS4* results in increased oxidative stress

As H_2O_2 metabolic process (GO:0042743), response to ROS (GO:0000302), and response to oxidative stress (GO:0006979) were enriched in rdm4 plants relative to the WT (Table 1), we then analyzed the detailed changes of ROS-related genes based on microarray data in this study. Cluster analysis indicated that many more ROS genes were changed in the C24 WT (184 genes, including 46 genes with twofold change) than in the *rdm4* mutant (145 genes, including 17 genes with twofold change) after short-term chilling treatment (3 h). For example, the expression level of two copper/zinc SODs (At1g08830 and At2g28190) showed significant increases only in the C24 WT (Table S4). Two peroxidases (At4g30170 and At5g17820) were induced more than 10-fold in the C24 WT, but only twofold in *rdm4* after the chilling treatment. After long-term chilling (48 h), the expression patterns of ROS genes in the WT were similar to that in *rdm4* plants (Fig. 5a; Table S4). Although chilling significantly increased H_2O_2 content in both Col and C24 WT plants, both *rdm4* and *dms4* mutants accumulated a greater amount of H_2O_2 than their respective WT parent plants after chilling (Fig. 5b). Consistent with the higher H_2O_2 content in the mutants, activities of both CAT and SOD were significantly higher in WT plants than in their mutants (Fig. 5c,d). These results indicated that mutations of *RDM4/DMS4* resulted in a reduced expression level and enzyme activity of antioxidants and thereby increased the accumulation of ROS, which might cause an increase in oxidative stress in *rdm4/dms4* plants under chilling.

Modulation of *RDM4* affects cold stress-related genes

*The CBF*s are key regulators during the plant cold stress response. We observed that most target genes of *CBF*s and *CBFs* showed reduced expression in the *rdm4* mutant line before and after chilling compared with WT plants (Fig. S3a; Table S5), but were constitutively expressed in the *RDM4* overexpressor before chilling (Fig. S3b; Table S5). To further characterize the putative connection between *RDM4* and the *CBF* pathway, we compared genes changed in the *rdm4* mutant and in the *CBF2* and *CBF3* overexpression lines reported by Vogel *et al.* (2005) and Chan *et al.* (2012). In total, 32–48 genes were regulated in both the *CBF2* transgenic lines and the *rdm4* mutant before and 3 and 48 h after chilling treatment, while many more genes (100–156) were affected in both the *CBF3* transgenic lines and the *rdm4* mutant with or without the chilling treatment (Fig. 6a,b). Most of these genes were regulated in the opposite directions by *CBF2 3* and *rdm4* (Fig. S4; Tables S6, S7). Quantitative real-time PCR was performed to verify microarray data and the results showed that the trends of both increased and decreased expression for selected genes were similar (Fig. 6c). Not surprisingly, about half of the genes (216–403) were affected by both the chilling treatment and the *rdm4* mutation (Fig. 6d, red square), which accounts for up to 72% of the genes changed after 3 h of chilling in rdm4 vs WT (Fig. 6d, blue square). For example, 331, 403 and 241 genes affected in the WT (after 3 h chilling vs room temperature) were also changed in the rdm4 mutant after 0, 3, and 48 h of chilling, respectively, and 247, 236 and 216 genes affected in the WT (after 48 h chilling vs room temperature) overlapped with those in the rdm4 vs WT comparisons after 0, 3 and 48 h chilling treatments, respectively. However, overexpression of *CBF2* or *CBF3* did not significantly change the expression level of *RDM4* (Table S8), which was induced after 3 h of chilling (Tables S1, S2), indicating *RDM4* is not a target of *CBF*s. These results indicate that modulation of *RDM4* significantly regulates cold stress-related genes in a *CBF*-dependent pathway.

RDM4 promotes the binding of Pol II to the promoter of *CBF* genes

To further characterize the roles of *RDM4* during transcription of cold stress-related genes, we performed ChIP assays using Pol II antibody in C24 WT and *rdm4* mutant lines and GFP antibody in *RDM4*-YFP ox lines and Col WT. As reported previously, RDM4 is associated with Pol II *in vivo* (He *et al.*, 2009). The Pol II ChIP assay indicated that Pol II was significantly enriched at the *CBF2* and *CBF3* promoters after 3 h at 4°C in both *rdm4* and C24 WT. However, mutation of *RDM4* significantly decreased the occupancy of Pol II at the *CBF2* and *CBF3* promoters, and weakly decreased the occupancy of Pol II at the *CBF1* promoter, as compared with the C24 WT. Enrichment of Pol II at the *CBF3* promoter was fivefold higher in C24 WT than in the *rdm4* mutant (Fig. 7a). Moreover, only a slight enrichment was observed at the promoters of the *CBF* upstream regulators, including *ICE1*, *CAMTA3*, *CCA1*, and *LHY* and cold-inducible *At1g02820*. To determine whether *RDM4* directly binds to promoters of earlier mentioned genes, we created YFP-tagged *RDM4* overexpression lines and performed Pol II and GFP ChIP assays. Pol II ChIP data showed a significant enrichment at the promoters of *CBF2* and *CBF3* before cold treatment, and a slight enrichment at the promoters of *CBF1*, *CCA1* and *LHY* (Fig. 7b). The results showed no significant enrichment for these genes despite a slight increase for *CBF3*, *ICE1*, *LHY* and *At1g02820* (Fig. 7c). They indicated that *RDM4* is important for the Pol II transcriptional complex to selectively activate transcription of the *CBF2* and *CBF3* genes.

Discussion

During the response of plants to abiotic stress, expressions of stress-responsive genes are regulated not only by gene-specific sequences and protein–DNA interactions but also by epigenetic events such as DNA methylation and dynamic histone modifications through acetylation, methylation, phosphorylation, and ubiquitinylation. Under stress conditions, genes may exhibit alternate epigenetic states, which result in differences in transcript abundance without changes in the primary DNA sequence (Lukens & Zhan, 2007; Chinnusamy & Zhu, 2009). Recent studies reported that the plant methylome is changed by abiotic stress and identified a group of stress-responsive genes that are potentially regulated by epigenetic mechanisms (Popova *et al.*, 2013). Mutation of *Arabidopsis HISTONE DEACTYLASE 6* (*HDA6*) caused hypersensitivity to ABA and to salt stress as well as reduced expression of ABA and abiotic stress-responsive genes (Chen *et al.*, 2010). *Arabidopsis* DNA demethylases, including *REPRESSOR OF SILENCING 1* (*ROS1*), *DEMETER-LIKE 2* (*DML2*) and *DML3*, play a role in fungal disease resistance in *Arabidopsis* (Le *et al.*, 2014). *DECREASE IN DNA METHYLATION1* (*DDM1*) and *ROS1* were reported to participate in UV-B-induced and oxidative DNA damage repair in *Arabidopsis* (Qüuesta *et al.*, 2013). In plants, Pol IV and Pol V evolved from Pol II but have distinct functions in the RdDM pathway. Pol IV is believed to produce single-stranded RNAs that serve as precursors of siRNAs. Pol V and Pol II, by contrast, are involved in producing noncoding RNA (ncRNA) scaffolds with which 24-nt sRNAs form base pairs. Mutation of *NRPD2*, the second largest common subunit of the Pol IV and Pol V complexes, and of *HDA6* rendered plants hypersensitive to acute heat stress (Chen *et al.*, 2010). All of these results indicate that epigenetic changes affect the response of plants to abiotic stress. *RDM4*/*DMS4* has important roles in epigenetic regulation through the RdDM pathway, and it also functions in the regulation of plant growth and development via Pol II transcription of protein-coding genes (He *et al.*, 2009; Kanno *et al.*, 2010; Law *et al.*, 2011).

As a major environmental stress, cold inhibits plant growth and development. *CBF*s/*DREB*s are key regulators in the response of plants to cold stress, and extensive studies have discovered downstream *CBF* regulon genes (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Fowler & Thomashow, 2002; Gilmour *et al.*, 2004). Research on the upsteam regulators of the *CBF* pathway identified four transcription factors – *ICE1*, *CAMTA3*, *CCA1*, and *LHY* – that bind directly to promoters of *CBF1*, *CBF2* and *CBF3* (15, 16, 20). Recent studies indicated that epigenetic regulation might also play an important role in plant acclimation to low temperatures (Chinnusamy & Zhu, 2009; Hu *et al.*, 2011, 2012). *CBF1* expression is activated through interaction with histone acetyltransferases such as GCN5 (Stockinger *et al.*, 2001). Hyperacetylation of the promoter region of *DREB1*/*CBF* in rice and maize during cold stress promotes gene transcription (Hu *et al.*, 2011; Roy *et al.*, 2014).

In a previous study (He *et al.*, 2009), we found that mutation of *RDM4* affects the expression level of several cold stress-responsive genes. These results prompted us to further characterize the roles of *RDM4* in the response of *Arabidopsis* to cold stress. Our data indicated that *rdm4* and *dms4* mutants are more sensitive to chilling and freezing treatments than their WT counterparts and that the RdDM pathway is not required for cold stress responses because *nrpe1* is not hypersensitive to chilling. *RDM4* overexpression reduces the

electrolyte leakage under chilling conditions (Fig. 3), but does not induce stronger expression of *CBF*s or *CBF* regulons under chilling conditions (Fig. 3b). There are two reasons why this might be the case. First, *CBF*s or *CBF* regulons were constitutively expressed in the *RDM4* overexpressor before the chilling treatment (Fig. 3b). Second, microarray data showed that many genes were not coregulated by *RDM4* and *CBF2*/*3* (Fig. 6). Therefore, other *CBF* independent pathways might be involved in the *RDM4*-regulated cold stress response. Moreover, the EL assay indicated that *RDM4* is required for tolerance to chilling conditions but does not play a role in basal freezing tolerance, as the difference in EL is visible in plants that have been chilled or acclimated but not in those that have been frozen directly. One possibility is that the association of RDM4 and Pol II is activated under chilling conditions, while the association might be disturbed when exposed to nonacclimated freezing conditions.

When exposed to abiotic stress, plants accumulate ROS, some of which may be important for activating the defense response. However, overproduction of ROS is damaging because ROS can oxidize proteins, damage nucleic acids, and cause lipid peroxidation (Mittler, 2002; Apel & Hirt, 2004). To scavenge overproduced ROS, plants have developed complex antioxidant defense systems, including antioxidant enzymes like SOD, CAT and peroxidase (POD). As described earlier, mutation of *RDM4*/*DMS4* greatly affected expression of *CBF* genes (Table S5). Overexpression of *CBF* genes resulted in extensive transcriptional reprogramming in *Arabidopsis* (Fowler & Thomashow, 2002; Vogel *et al.*, 2005) and caused changes in the ROS contents and antioxidant enzyme activities in *Arabidopsis*, tobacco, and tomato (Fowler & Thomashow, 2002; Yang *et al.*, 2010; Zhang *et al.*, 2011). In this study, *RDM4* modulates the expression level of *CBF* genes which might further regulate the ROS pathway. Consistently, modulation of *RDM4* affected the expression level of several ROS-related genes (Fig. 5; Table S4). These results indicated that *RDM4* might function directly or indirectly during an oxidative burst under stressed conditions, as evidenced by extensive changes of ROS-related genes and antioxidant enzyme activities, and the accumulation of high concentrations of H_2O_2 in the *rdm4* plant (Fig. 5; Table S4).

RDM4/DMS4 in plants and its ortholog Iwr1 in yeasts are associated with Pol II (He *et al.*, 2009; Peiró-Chova & Estruch, 2009; Kanno *et al.*, 2010; Czeko *et al.*, 2011; Esberg *et al.*, 2011). Iwr1 contains a nuclear localization signal and has been shown to help shuttle the Pol II complex from the cytoplasm into the nucleus (Czeko *et al.*, 2011; Esberg *et al.*, 2011). Our previous work found that the nucleoporin *AtNUP160* is critical for cold-responsive gene expression and for chilling tolerance and acquired freezing tolerance (Dong *et al.*, 2006). It is possible that the nuclear import of the Pol II complex may be very sensitive to cold temperatures and that *RDM4* is of particular importance for the import of Pol II complex under cold stress conditions. Alternatively, *RDM4* may have functions during the nuclear import of Pol II, and may regulate Pol II activity under specific conditions. Our ChIP assay showed that RDM4 is important for Pol II occupancy at the promoters of *CBF* genes when *rdm4* and C24 WT lines were used (Fig. 7a). Overexpression of *RDM4* (*RDM4*-YFP line) resulted in significant enrichment of Pol II at the promoter of *CBF2* and *CBF3* as compared with the WT before cold treatment (Fig. 7b). These results were consistent with microarray data showing that overexpression of *RDM4* significantly increased the gene expression level before cold treatment (Fig. S3). Therefore, it is possible that overexpression of *RDM4*

resulted in significantly higher Pol II occupancy even without cold treatment. RDM4 may also be important for Pol II occupancy at the promoters of other cold-induced genes that are affected by the *rdm4* mutation. However, as not all cold-induced genes are affected by *rdm4*, RDM4 may specifically promote Pol II occupancy at the promoters of part of cold-induced genes. Further studies to elucidate detailed mechanisms of putatively post-translational modifications of the RDM4 protein will provide additional evidence for *RDM4*-regulated plant cold stress responses. In a previous study, we found evidence that *rdm4* suppresses transcriptional gene silencing of *RD29A-LUC* and endogenous *RD29A* in ros1 by blocking DNA hypermethylation at the *RD29A* promoter (He *et al.*, 2009). Whether DNA methylation or other chromatin modifications in *rdm4* or *RDM4* overexpressor affect Pol II occupancy, or binding activities of transcription factors such as *ICE1* and *CAMTA3* in the promoters of *CBF* genes, remains to be elucidated and further studies in this respect are needed.

Overall, our results and those of others suggest that by interacting with Pol II, RDM4 may then promote binding of Pol II to *CBF2/3* promoters under cold stress conditions and up-regulate the expression of *CBF*s and *CBF* downstream regulon genes. *RDM4* also modulates ROS metabolism to minimize damage caused by redox stress. Our results suggest that *RDM4* has a considerable role in regulating gene expression at low temperatures, including the *CBF* pathway, resulting in improved cold stress tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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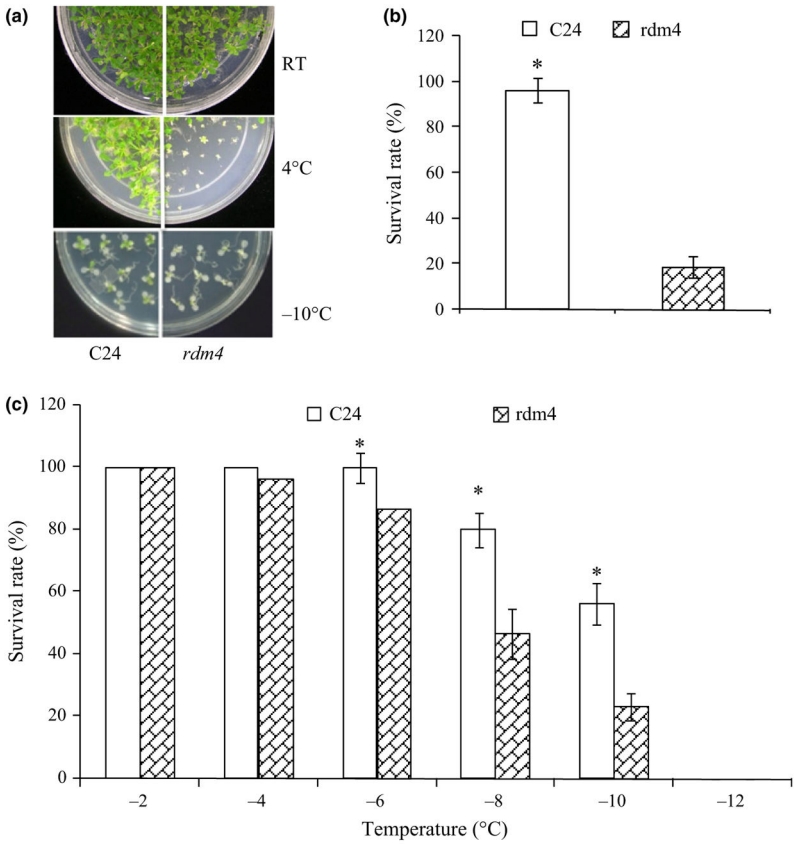


Fig. 1.

The *Arabidopsis rdm4* mutant is sensitive to chilling and freezing stresses. (a) Phenotypes of *rdm4* and the C24 wild-type (WT) at room temperature (RT) and under chilling and freezing stress conditions. (b) Survival rate of *rdm4* and the C24 WT after 4 months at 4°C. (c) Survival rate of *rdm4* and the C24 WT after freezing treatment. Chilling was initiated at 48 h after planting. For the freezing treatment, 10-d-old seedlings were cold-acclimated for 4 d before being moved to the growth chamber which was programmed to cool at $1°Ch^{-1}$ as described in the Materials and Methods section. Values are means ± SE ($n = 3$) and the asterisks indicate a significant difference relative to the *rdm4* mutant.

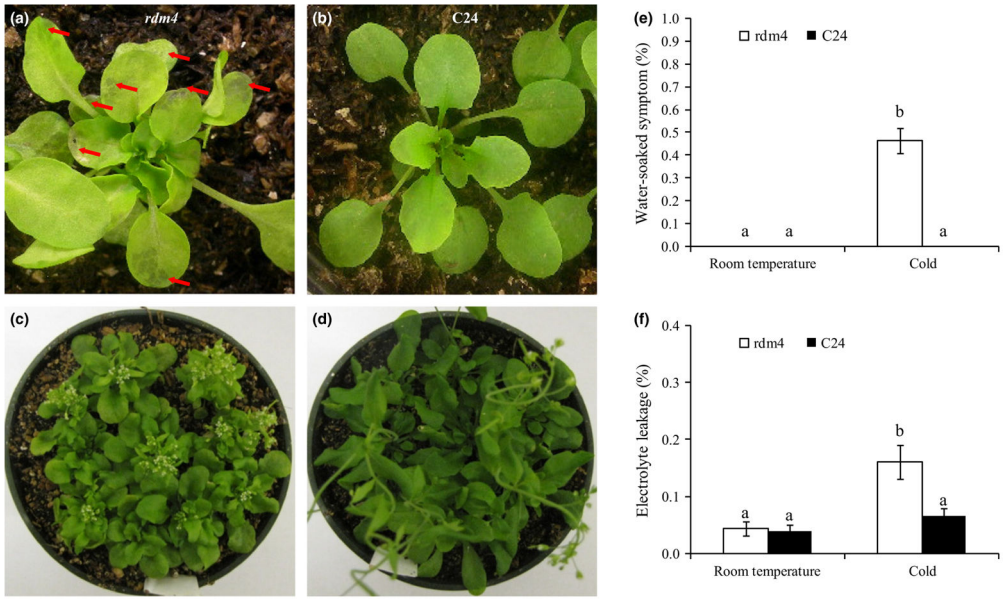


Fig. 2.

Transparent leaf symptoms of *Arabidopsis rdm4* mutant under chilling conditions. Two-week-old plants were transferred to a cold room preset at 4°C. (a, b) Room temperature-cultivated plants were used as controls. (c) Transparent leaf symptoms (red arrows) appeared in the rdm4 mutant after 4 wk of the chilling treatment, but there were none in wild-type plants (d). (e) Percentage of water-soaked symptoms of C24 and rdm4 plants. (f) Electrolyte leakage of C24 and rdm4 plants. Values are means ± SE ($n = 3$). Values followed by different letters are significantly different at $P < 0.05$ according to Duncan's test.

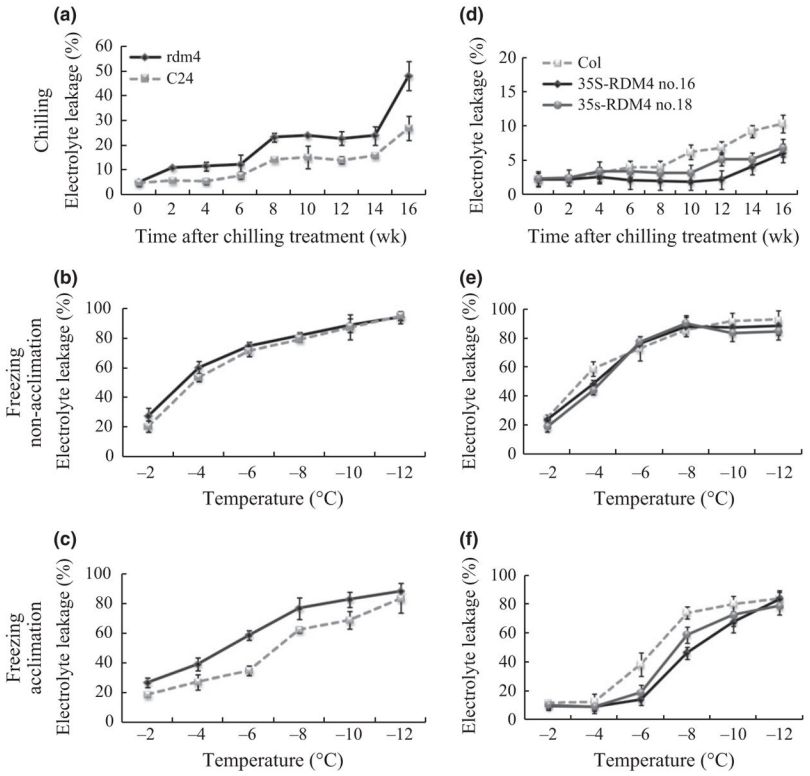


Fig. 3.

Changes in electrolyte leakage (EL) values after chilling and freezing treatments in the *Arabidopsis rdm4* mutant and the wild-type (WT). (a–c) EL values of the *rdm4* mutant and the C24 WT. (d–f) EL values of *RDM4* overexpression lines and the Col WT. Values are means ± SE ($n = 3$). For EL assay under chilling conditions, 2-wk-old plants were subjected to 4°C treatment for the indicated time points. For EL assay under freezing conditions, one excised leaflet from 3-wk-old plants with or without 4 d of cold acclimation was used as described in the Materials and Methods section. Values are means ± SE ($n = 3$).

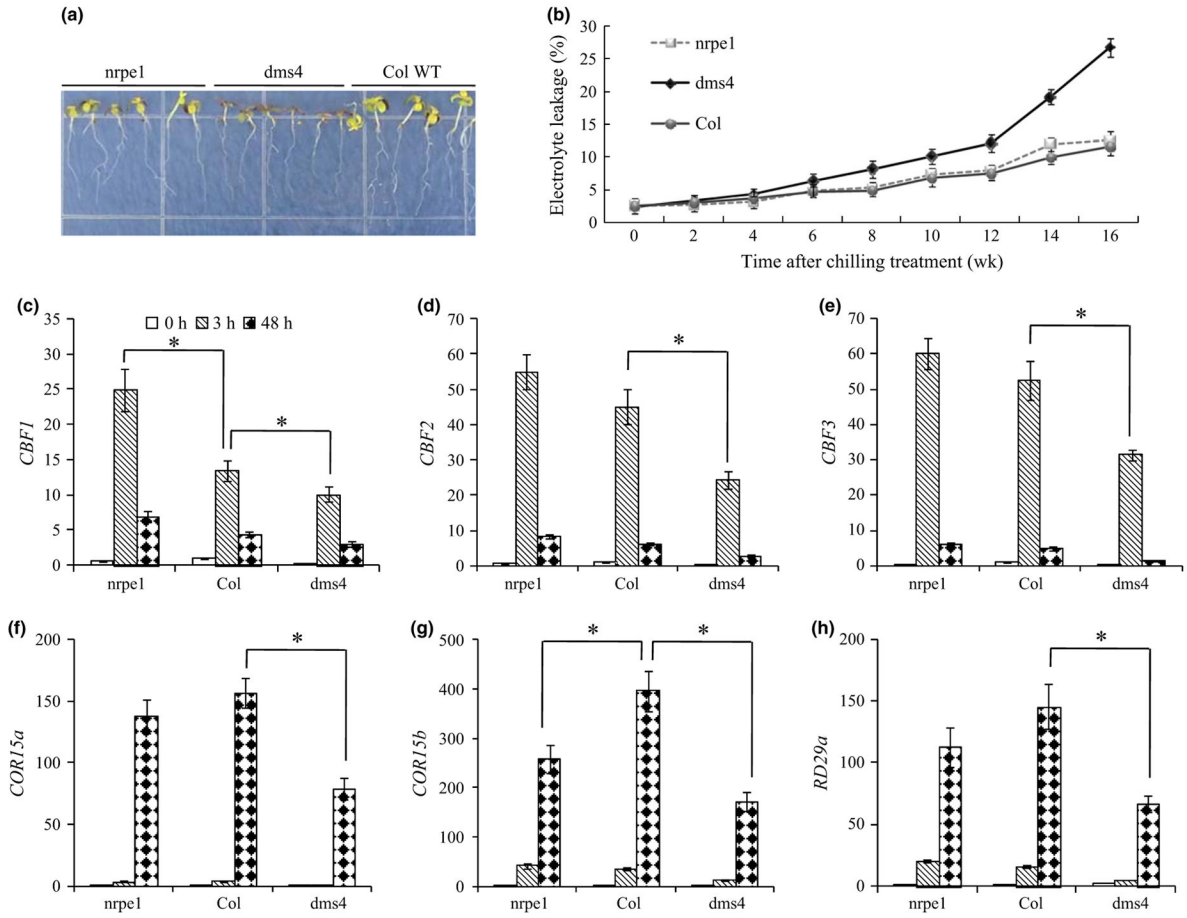


Fig. 4.

Arabidopsis dms4 seedlings, but not *nrpel* seedlings, are sensitive to chilling. (a) Phenotypes of *nrpe1*, *dms4* and Col wild-type (WT) after 4 months at 4°C. (b) Electrolyte leakage values of three genotypes incubated at 4°C for the indicated times. (c–h) Relative expression level of *CBF* and *CBF* regulon genes in the three genotypes incubated at 4°C for the indicated times. Expression levels in the Col WT at 0 h were normalized to 1 and all other data were relative to Col at 0 h. Values are means ± SE ($n = 3$) and the asterisks indicate a significant difference between the mutants and Col WT.

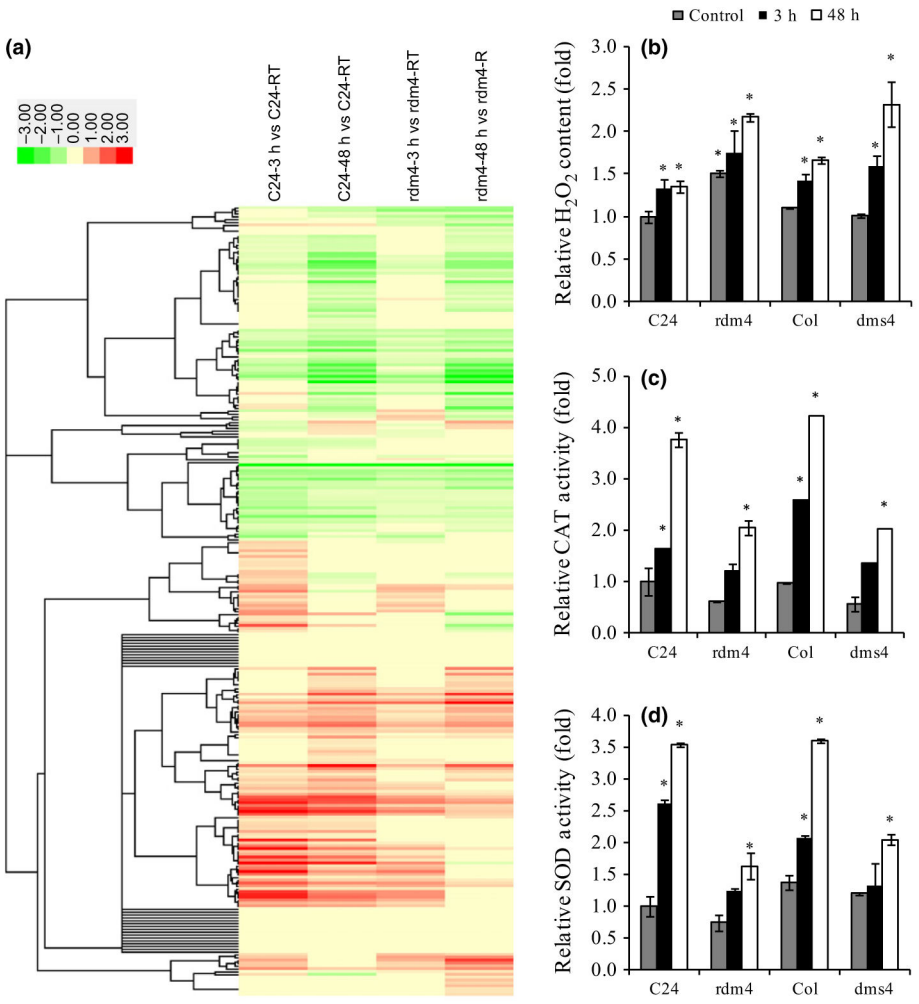
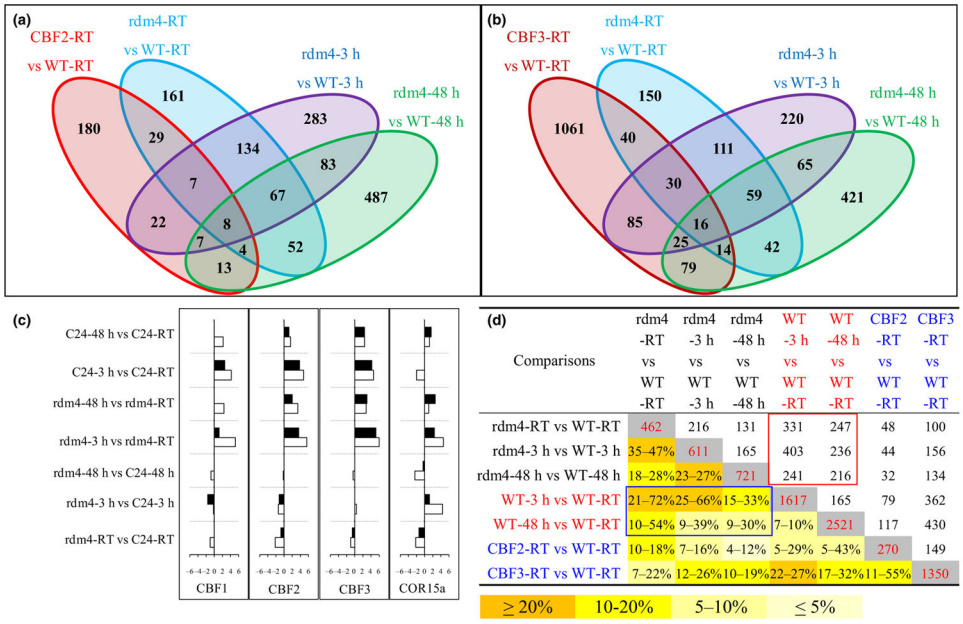


Fig. 5.

Mutation of *rdm4*-affected reactive oxygen species (ROS) homeostasis in *Arabidopsis*. (a) Cluster analysis of expression levels of antioxidant-related genes from microarray analysis in this study. (b) Hydrogen peroxide (H_2O_2) contents. (c) Enzyme activity of catalase (CAT). (d) Enzyme activity of superoxide dismutase (SOD). The detailed ROS gene expression data are listed in Table S4. Values are means ± SE ($n = 3$) and the asterisks indicate a significant difference relative to the room temperature (RT) control.



(d)

Comparisons	rdm4 -RT vs WT -RT	rdm4 -3 h vs WT -3 h	rdm4 -48 h vs WT -48 h	WT -3 h vs WT -RT	WT -48 h vs WT -RT	CBF2 -RT vs WT -RT	CBF3 -RT vs WT -RT
rdm4-RT vs WT-RT	462	216	131	331	247	48	100
rdm4-3 h vs WT-3 h	35–47%	611	165	403	236	44	156
rdm4-48 h vs WT-48 h	18–28%	23–27%	721	241	216	32	134
WT-3 h vs WT-RT	21–72%	25–66%	15–33%	1617	165	79	362
WT-48 h vs WT-RT	10–54%	9–39%	9–30%	7–10%	2521	117	430
CBF2-RT vs WT-RT	10–18%	7–16%	4–12%	5–29%	5–43%	270	149
CBF3-RT vs WT-RT	7–22%	12–26%	10–19%	22–27%	17–32%	11–55%	1350

≥ 20% | 10-20% | 5–10% | ≤ 5%

Fig. 6.

Overlapping analysis of *rdm4*-affected genes with *CBF2* or *CBF3* altered genes in *Arabidopsis*. *CBF2* and *CBF3* microarray data were downloaded from the NCBI GEO database with the accession numbers GSE5536 and GSE26983, respectively. (a) Number of overlapping genes between *RDM4* and *CBF2*. (b) Number of overlapping genes between *RDM4* and *CBF3*. (c) Verification of microarray data (open bars) using quantitative real-time PCR (closed bars). (d) Percentage of overlapping genes. The gray background indicate the number of genes changed by each comparison. Numbers in the upper triangle with the white background indicate absolute numbers of overlapping genes. Numbers in the lower triangle with the orange/yellow background are percentages of overlapping genes, calculated by dividing the number of overlapping genes (upper triangle) by the number of changed genes in each comparison (grey background). WT, wild-type; RT, room temperature. '−3h' and '−48h' appended to plant types indicate after 3 and 48 h of chilling, respectively.

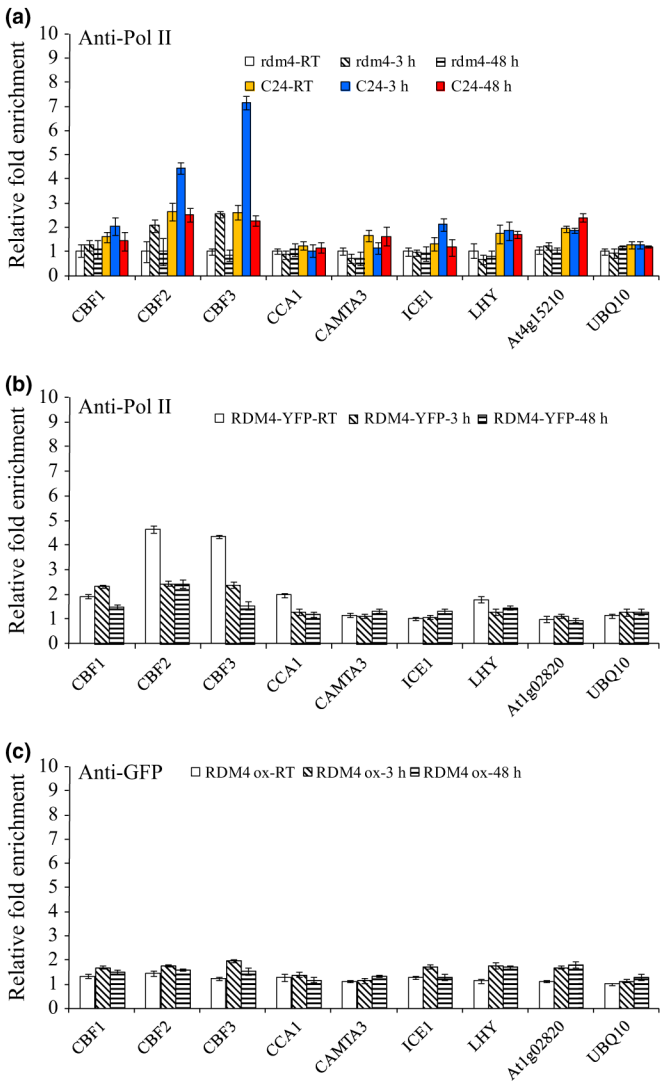


Fig. 7.

RDM4 protein is important for RNA polymerase II (Pol II) occupancy at the promoters of *CBF* genes in *Arabidopsis*. (a) Immunoprecipitation (IP) with Pol II antibody in rdm4 and the wild-type. (b) IP with Pol II antibody in yellow fluorescence protein (YFP)-tagged *RDM4* overexpression lines. (c) IP with green fluorescence protein (GFP) antibody in YFP-tagged *RDM4* overexpression lines. The chromatin IP (ChIP) results were normalized to input chromatin, and a fragment in the *UBQ10* promoter was used as the negative control. Expression level of *UBQ10* in *rdm4* at room temperature (RT) (a) or C24 at RT (b, c) was normalized to 1 and all other data were relative to *UBQ10*. *CCA1*, *CAMTA3*, *ICE1* and *LHY* are upstream regulators of *CBF*s. Chilling-induced *At1g02820* is not a *CBF* regulon. The detailed primers used for ChIP-PCR are listed in Table S8. The data represent means ± SD of three independent experiments. For each gene, values followed by different letters within each panel are significantly different at $P < 0.05$ according to Duncan's test. '−3h' and '−48h' appended to plant types indicate after 3 and 48 h of chilling, respectively.

Table 1

Gene ontology (GO) term enrichment analysis of genes altered in *Arabidopsis rdm4* lines and by cold stress treatment

GO ID	Term	rdm4-RT vs C24-RT	rdm4-3h vs C24-3 h	rdm4-48h vs C24-48 h	C24-3h vs C24-RT	C24-48h vs C24-RT
GO:0019915	Lipid storage	3.72	/	/	/	/
GO:0010876	Lipid localization	3.16	2.42	1.92	1.53	/
GO:0042743	Hydrogen peroxide metabolic process	3.07	2.77	/	2.21	/
GO:0072593	Reactive oxygen species metabolic process	3.07	2.67	/	2.17	1.62
GO:0080134	Regulation of response to stress	2.83	/	2.81	1.61	/
GO:0010243	Response to organic nitrogen	2.76	1.77	3.05	1.64	1.10
GO:0000302	Response to reactive oxygen species	2.52	2.46	/	1.93	1.25
GO:0009611	Response to wounding	2.44	2.04	2.01	1.81	1.35
GO:1901698	Response to nitrogen compound	2.41	/	2.62	1.40	1.00
GO:0044550	Secondary metabolite biosynthetic process	2.35	2.51	/	1.75	1.49
GO:0006979	Response to oxidative stress	2.35	1.95	1.81	1.63	1.15
GO:0009698	Phenylpropanoid metabolic process	2.18	2.39	/	1.79	1.61
GO:0019748	Secondary metabolic process	2.17	2.32	/	1.67	1.37
GO:0009617	Response to bacterium	2.01	1.54	2.02	/	/
GO:1901700	Response to oxygen-containing compound	1.94	1.55	1.77	1.26	0.82
GO:0009409	Response to cold	1.90	1.88	1.59	1.09	1.04
GO:0014070	Response to organic cyclic compound	1.78	1.46	1.54	/	/
GO:0097305	Response to alcohol	1.77	/	1.65	0.99	0.63
GO:0033993	Response to lipid	1.74	/	1.50	0.99	/
GO:0051707	Response to other organism	1.65	1.62	1.75	1.14	0.64
GO:0010033	Response to organic substance	1.56	1.10	1.69	1.03	0.47
GO:0033554	Cellular response to stress	1.53	1.47	/	0.88	/
GO:0006970	Response to osmotic stress	1.47	/	/	0.73	0.86
GO:0009725	Response to hormone stimulus	1.41	1.01	1.43	1.07	0.47
GO:0010035	Response to inorganic substance	1.41	1.54	/	1.05	1.06
GO:0070887	Cellular response to chemical stimulus	1.36	1.00	1.07	1.09	0.62
GO:0009266	Response to temperature stimulus	1.33	1.61	1.52	0.96	1.10
GO:0009651	Response to salt stress	1.29	/	/	/	0.85

GO ID	Term	rdm4-RT vs C24-RT	rdm4-3h vs C24-3 h	rdm4-48h vs C24-48 h	C24-3h vs C24-RT	C24-48h vs C24-RT
GO:0055114	Oxidation-reduction process	1.09	1.07	/	0.88	/
GO:0044248	Cellular catabolic process	1.06	1.11	/	0.61	0.60
GO:0009308	Amine metabolic process	/	2.04	/	/	1.14
GO:1901565	Organonitrogen compound catabolic process	/	1.81	/	1.41	1.06
GO:0005976	Polysaccharide metabolic process	/	1.46	/	1.15	/
GO:0008219	Cell death	/	/	1.80	/	/

Genes with *P*-value ≤ 0.05 and fold change ≥ 2.0 were analyzed using GO$_{EAST}$. Log odds ratio were presented and calculated as described in the Materials and Methods section. /, indicates that the GO term is not significantly enriched. RT, room temperature; '−3h' and '−48h' appended to plant types indicate after 3 and 48 h of chilling, respectively.