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A DEAD Box RNA Helicase Is Critical for Pre-mRNA Splicing, Cold-Responsive Gene Regulation, and Cold Tolerance in *Arabidopsis*

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Cold stress resulting from chilling and freezing temperatures substantially reduces crop production worldwide. To identify genes critical for cold tolerance in plants, we screened *Arabidopsis thaliana* mutants for deregulated expression of a firefly *luciferase* reporter gene under the control of the *C-REPEAT BINDING FACTOR2* (*CBF2*) promoter (*CBF2:LUC*). A *regulator of CBF gene expression1* (*rcf1-1*) mutant that is hypersensitive to cold stress was chosen for in-depth characterization. *RCF1* encodes a cold-inducible DEAD (Asp-Glu-Ala-Asp) box RNA helicase. Unlike a previously reported DEAD box RNA helicase (LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES4 [LOS4]) that regulates mRNA export, RCF1 does not play a role in mRNA export. Instead, RCF1 functions to maintain proper splicing of pre-mRNAs; many cold-responsive genes are mis-spliced in *rcf1-1* mutant plants under cold stress. Functional characterization of four genes (*PSEUDO-RESPONSE REGULATOR5* [*PRR5*], *SHAGGY-LIKE SERINE/THREONINE KINASE12* [*SK12*], *MYB FAMILY TRANSCRIPTION FACTOR CIRCADIAN1* [*CIR1*], and *SPFH/PHB DOMAIN-CONTAINING MEMBRANE-ASSOCIATED PROTEIN* [*SPFH*]) that are mis-spliced in *rcf1-1* revealed that these genes are cold-inducible positive (*CIR1* and *SPFH*) and negative (*PRR5* and *SK12*) regulators of cold-responsive genes and cold tolerance. Together, our results suggest that the cold-inducible RNA helicase RCF1 is essential for pre-mRNA splicing and is important for cold-responsive gene regulation and cold tolerance in plants.

INTRODUCTION

Adaptation is the way of life for sessile and poikilothermic land plants, which must endure environmental stresses, including those caused by low/high temperatures, water deficit, and salinity. These abiotic stresses not only limit the geographical distribution of plants but also reduce the global productivity and quality of important agricultural crops. Although plant temperature changes with the ambient temperature, most temperate plants can acquire tolerance to freezing temperatures by a prior exposure to low nonfreezing temperatures, a process termed cold acclimation (Guy, 1990; Thomashow, 1999; Ruelland et al., 2009). Freezing tolerance is essential for temperate crops like winter wheat (*Triticum aestivum*) and canola (*Brassica napus*), but tropical and subtropical plants are incapable of cold acclimation. Thus, the productivity and quality of tropical crops (like rice [*Oryza sativa*], maize [*Zea mays*], soybean [*Glycine max*], cotton [*Gossypium hirsutum*], and tomato [*Solanum lycopersicum*]) are reduced even by nonfreezing low temperatures (i.e., chilling). Therefore, the engineering or breeding of chilling- and freezing-tolerant crop plants is an important goal in agriculture. Such engineering or breeding requires a thorough understanding of the molecular mechanisms of cold stress signal perception and transduction in plant cells that lead to chilling tolerance and/or cold acclimation.

The expression of many genes in plants is regulated by low temperature (Dhindsa and Monroy, 1994; Guy et al., 1994; Palva et al., 1994; Thomashow, 1994). Most of these genes maintain high levels of expression throughout cold treatment, but their expression decreases rapidly upon return from cold to normal growth temperatures (Thomashow, 1994). The cold-responsive genes encode a diverse array of proteins, including enzymes involved in respiration and in the metabolism of carbohydrates, lipids, phenylpropanoids, and antioxidants; molecular chaperones; antifreezing proteins; and many other proteins of unknown function (Guy et al., 1994).

Differential screening and cloning studies in plants have identified a core set of robustly cold-regulated genes (Thomashow, 1999). The promoters of many of these genes contain one or several copies of the dehydration-responsive element (*DRE*)/C-repeat (*CRT*) *cis*-element, which has the core sequence CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). CRT binding factors (CBFs), also known as DRE binding proteins, are upstream transcription factors in the APETALA2/ETHYLENE RESPONSE FACTOR family that bind to the promoter *cis*-element and activate the expression of these cold-responsive genes (Stockinger et al., 1997; Liu et al., 1998). The *CBF* genes themselves are induced by low temperatures, and this induction is transient and precedes that of downstream

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cold-responsive genes with the *DRE/CRT cis*-element (Medina et al., 1999). Transgenic *Arabidopsis thaliana* plants ectopically expressing *CBF3* contain elevated levels of Pro and soluble sugars, which correlate with increased freezing tolerance (Gilmour et al., 2000). INDUCER OF *CBF* EXPRESSION1 (ICE1), a basic helix-loop-helix protein, is an upstream transcription factor that binds to the *CBF3* promoter and is required for activation of *CBF3* expression upon cold stress (Chinnusamy et al., 2003). An R2R3-type MYB transcription factor, MYB15, interacts with ICE1 and negatively regulates the expression of *CBF* genes under cold stress (Agarwal et al., 2006). HOS1, a negative regulator of the *CBF2* and *CBF3* genes, was identified from a genetic screen for mutants with enhanced expression of *CBF* target genes (Ishitani et al., 1998; Lee et al., 2001). *HOS1* encodes a RING finger protein that has ubiquitin E3 ligase activity (Lee et al., 2001; Dong et al., 2006). Both in vitro and in vivo ubiquitination assays showed that HOS1 mediates the polyubiquitination of ICE1 under cold conditions (Dong et al., 2006). *SENSITIVE TO FREEZING6* plays a role in cold acclimation via the CBF pathway after CBF translation (Knight et al., 2009). Recently, Shi et al. (2012) reported that proteins in the ethylene signaling pathway negatively regulate freezing tolerance by downregulation of *CBF* and type-A *Arabidopsis RESPONSE REGULATOR* genes in *Arabidopsis*.

CBF2 negatively regulates CBF1 and CBF3 (Novillo et al., 2004), and Zn TRANSPORTER OF *Arabidopsis thaliana12* (ZAT12; a C2H2 zinc finger protein) negatively regulates the expression of the *CBF* genes (Vogel et al., 2005). Novillo et al. (2007) reported that CBF1 and CBF3 function additively in cold acclimation and differently from CBF2; CBF2 defines different gene classes in the CBF regulon. Doherty et al. (2009) showed that members of calmodulin binding proteins of the CAMTA family of transcription factors can bind to one of the *cis*-elements in the *CBF2* promoter in vitro and are important for freezing tolerance. Additional molecular factors modulating *CBF2* expression remain to be identified.

To identify additional molecular factors that are critical for cold tolerance and cold-responsive gene regulation, we fused the *CBF2* promoter to a firefly *luciferase* reporter gene (*CBF2*:*LUC*) and introduced this cold-inducible gene cassette into *Arabidopsis* plants. Screening of the ethyl methanesulfonate (EMS)–mutagenized M2 plants led to the isolation of mutants with altered *CBF* gene expression and/or cold stress tolerance. We designated these mutants as *regulator of CBF gene expression* (*rcf*). We report the in-depth characterization of one such mutant, *rcf1-1*. The *rcf1-1* mutant plants are hypersensitive to chilling and freezing temperatures. Map-based cloning revealed that *RCF1* encodes a cold-inducible DEAD (Asp-Glu-Ala-Asp) box RNA helicase. Unlike a previously reported DEAD box RNA helicase (LOS4) that regulates mRNA export, RCF1 maintains proper splicing of pre-mRNAs of nuclear-encoded genes. Loss-of-function and gain-of-function characterization of four genes that are mis-spliced in *rcf1-1* under cold stress revealed that they are important regulators of cold-responsive genes and cold tolerance. Together, our results indicate that a cold-inducible nuclear-localized RNA helicase RCF1 is critical for pre-mRNA splicing and for cold-responsive gene regulation and cold tolerance in plants.

RESULTS

Isolation of the *rcf1-1* Mutant

To identify novel molecular factors that regulate the expression of cold-responsive transcription factors and that have an essential role in cold tolerance, we generated transgenic *Arabidopsis* plants that express a firefly *LUC* reporter gene under the control of the cold-responsive *CBF2* promoter (*CBF2*:*LUC*, referred to as the wild type) and subsequently screened the EMS-mutagenized M2 population for mutants with deregulated *CBF2*:*LUC* expression. We designated these mutants as *rcf*. The *rcf1-1* mutant was chosen for in-depth characterization. Compared with wild-type plants, *rcf1-1* plants have a much higher level of *CBF2*:*LUC* expression under cold stress (Figures 1A and 1B), suggesting that RCF1 might be a negative regulator of *CBF2* gene expression.

We backcrossed *rcf1-1* with the wild type. All F1 plants showed a wild-type phenotype in response to cold stress, and F2 plants segregated at ~3:1 (wild type versus *rcf1-1*; see Supplemental Table 1 online). These results suggested that *rcf1-1* is a recessive mutation in a single nuclear gene.

RCF1 Is Required for Plant Tolerance to Chilling and Freezing Stresses

We investigated the effect of the *rcf1-1* mutation on plant sensitivity to chilling and freezing stresses. Chilling tolerance was assessed based on hypocotyl elongation in the dark. As shown in Figures 1C and 1D, hypocotyl elongation was similar for *rcf1-1* and the wild type at 21°C but was dramatically less for *rcf1-1* than for the wild type at 4°C, indicating that normal function of RCF1 is required for chilling stress tolerance. We subsequently determined the freezing tolerance of *rcf1-1* by two methods: an electrolyte leakage assay (Sukumaran and Weiser, 1972; Ishitani et al., 1998) and a whole-plant freezing assay (Warren et al., 1996; Jaglo-Ottosen et al., 1998; Xin and Browse, 1998; Zhu et al., 2008). The *rcf1-1* plants were hypersensitive to freezing temperatures before and after cold acclimation (Figures 1E to 1G), indicating that the ability to be fully acclimated is substantially reduced in *rcf1-1*. Together, these results suggest that RCF1 is a positive regulator of chilling and freezing tolerance in plants.

RCF1 Regulates Gene Expression under Cold Stress

To gain insight into the molecular function of RCF1 in the cold stress tolerance pathway, we examined its role in gene regulation with quantitative real-time RT-PCR (qRT-PCR) analysis and whole-genome microarray analysis. Consistent with the increased *CBF2*:*LUC* expression in *rcf1-1*, qRT-PCR analysis revealed that transcripts of *LUC* and endogenous *CBF2* were more abundant in *rcf1-1* at all time points after cold treatment (Figures 2A and 2B). We then determined whether the *rcf1-1* mutation affects other members in the *CBF* gene family. Expression of *CBF1* and *CBF3* was dramatically elevated in *rcf1-1* compared with the wild type throughout cold treatment (Figure 2C). CBF members are known to regulate the expression of downstream genes that have the DRE/C-repeat *cis*-element in

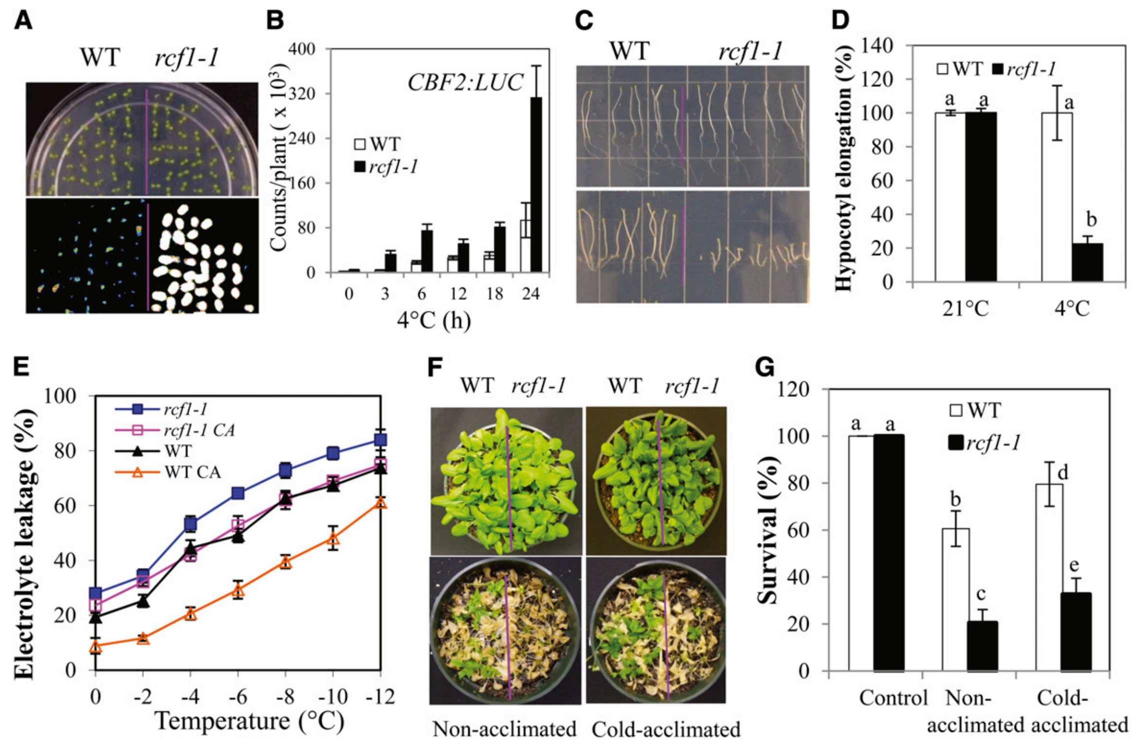

Figure 1. RCF1 Is a Positive Regulator of Cold Tolerance.

(A) *CBF2*:*LUC* expression in 7-d-old wild-type (WT) and *rcf1-1* seedlings treated at 4°C for 24 h.

(B) Quantification of luminescence intensity in **(A)**. *CBF2*:*LUC* expression was quantitatively measured as luminescence intensity (counts/plant). Data are also included for nonstressed and cold-treated seedlings at different time points.

(C) Chilling sensitivity of the wild type and *rcf1-1* at 4°C in darkness. Top panel, 21°C for 15 d; bottom panel, 4°C for 52 d.

(D) Quantification of hypocotyl elongation of plants shown in **(C)**. Hypocotyl elongation of the wild type at 21°C or 4°C was treated as 100%.

(E) Leakage of electrolytes from excised leaflets of *rcf1-1* and wild-type plants when treated at temperatures below freezing. *rcf1-1* CA, cold-acclimated *rcf1-1* plants; WT CA, cold-acclimated wild-type plants.

(F) Whole-plant freezing tolerance of the wild type and *rcf1-1*. Plants were photographed 10 d after freezing treatments.

(G) Survival rates of *rcf1-1* and the wild type in **(F)**.

Error bars represent the SD (n = 20 to 40 in **[B]**, 60 to 100 in **[D]**, 8 to 16 in **[E]**, and 100 to 140 in **[G]**). One-way analysis of variance (ANOVA; Tukey-Kramer test) was performed for data in **(D)** and **(G)**, and statistically significant differences are indicated by different lowercase letters ($P < 0.003$). Experiments in were repeated at least three times with similar results, and values shown are from one experimental repetition.

their promoters. Therefore, we examined whether *rcf1-1* affects the expression of known *CBF* downstream genes, such as *RD29A* and *COR15A*. Cold induction of *RD29A* and *COR15A* was substantially greater in *rcf1-1* than in wild-type plants (Figure 2D). These results, which sharply contrast with the reduced cold tolerance of the *rcf1-1* mutant (Figures 1C to 1G), indicate that RCF1 is a negative regulator of the expression of *CBF* genes and their downstream target genes under cold stress. These results also suggest that CBF-independent factors may not function properly in the *rcf1-1* mutant plants. Alternatively, increased expression of *CBF* genes in *rcf1-1* may be a compensatory response to the severely reduced cold tolerance of *rcf1-1*.

To detect changes in global gene expression in the *rcf1-1* mutant plants, we performed a whole-genome microarray analysis with Affymetrix *Arabidopsis* ATH1 GeneChips. RNA was extracted from both wild-type and *rcf1-1* seedlings that had been treated at 4°C for 0, 12, or 24 h. Analyses of the ATH1 microarray data indicated that, compared with their expression in wild-type seedlings, 35 genes were upregulated by at least threefold and five genes were downregulated by at least threefold in the *rcf1-1* mutant under control conditions (see Supplemental Table 2 online). Among the 35 upregulated genes in *rcf1-1*, most encode proteins that are predicted to function in response to stress (see Supplemental Table 2A online). The ATH1 microarray data also showed that after a 12-h cold treatment and compared with the wild type, *rcf1-1* contains 49 genes whose expression is significantly increased by at least threefold and 32 genes whose expression is significantly reduced by at least threefold (see Supplemental Data Set 1 online). After a 24-h cold treatment and compared with the wild type, expression in *rcf1-1* was increased by at least threefold for 95 genes and decreased by at least threefold for 73 genes (see Supplemental Data Set 2 online). Compared with their expression in the wild type, the expression of five genes was significantly increased in *rcf1-1* by at least threefold at all time points after cold treatment, while expression

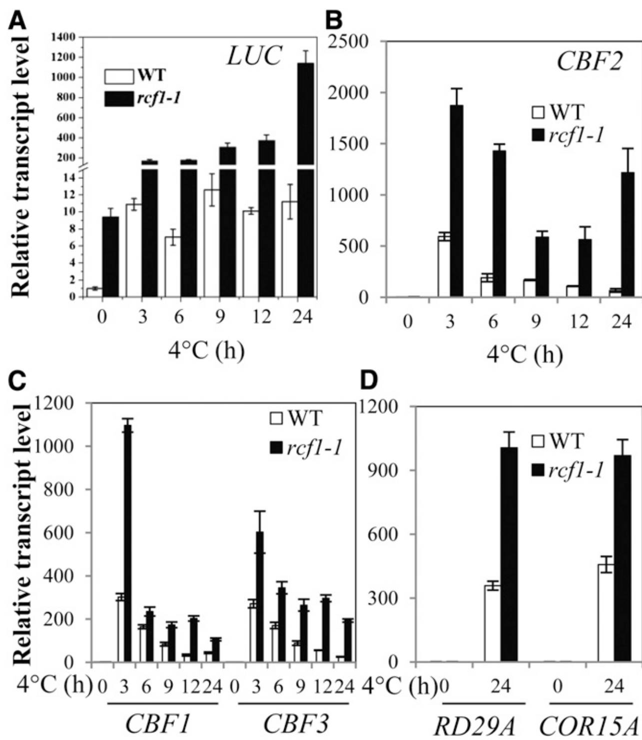

Figure 2. The *rcf1-1* Mutation Causes Higher Induction of the *LUC* Gene and Endogenous Cold-Responsive Genes under Cold Stress.

Transcript levels of the *LUC* gene and of endogenous *CBF2*, *CBF1*, *CBF3*, *RD29A*, and *COR15A* in 14-d-old wild-type (WT) and *rcf1-1* seedlings treated at 4°C for the indicated time periods. Error bars represent the SD (n = 4). Experiments in were repeated at least three times with similar results, and values shown are from one experimental repetition.

of one gene was significantly reduced in *rcf1-1* by at least threefold at all time points after cold treatment (see Supplemental Tables 3A and 3B online). Microarray data analysis also revealed that transcripts of 20 genes were significantly increased in *rcf1-1* by at least threefold after 12- and 24-h cold treatment, while transcripts of six genes were dramatically reduced in *rcf1-1* by at least threefold after 12- and 24-h cold treatment (see Supplemental Tables 3C and 3D online). Although the genes in *rcf1-1* that are differentially expressed in response to cold stress encode proteins involved in diverse biological processes, a relatively large proportion of the upregulated genes and some of the downregulated genes encode proteins that are predicted to function in biotic or abiotic stress response pathways (see Supplemental Table 3 and Supplemental Data Sets 1 and 2 online). Relative to publically available data concerning gene expression in response to cold stress in wild-type plants, gene expression substantially differs in *rcf1-1* (see Supplemental Figures 1 to 5 online).

In our ATH1 microarray analysis, transcripts of *CBF1*, *CBF2*, and *CBF3* were significantly increased in *rcf1-1* after 12 or 24 h of cold stress, suggesting that our microarray data are reliable. Our microarray analysis was confirmed by qRT-PCR analysis of seven genes that showed altered expression patterns in *rcf1-1* (Figure 2D; see Supplemental Figures 6A to 6D online). For example, both microarray analysis and qRT-PCR indicated that *ZAT12* in *rcf1-1* is upregulated after 12 and 24 h of cold treatment (see Supplemental Figure 6A online). Similarly, the detection of upregulation and downregulation was consistent for ATH1 microarray analysis and qRT-PCR for *At5g59950*, *Stabilized 1*, and *At2g42270*, which encode an RNA binding protein, a pre-mRNA splicing factor (STA1) (Lee et al., 2006), and a U5 small nuclear ribonucleoprotein helicase (Brr2b), respectively (see Supplemental Figures 6B and 6C online). Both ATH1 microarray and qRT-PCR analyses also indicated that one of the upstream transcriptional activators of *CBF2*, the CAMTA family protein *CAMTA1*, is downregulated in *rcf1-1* (see Supplemental Data Set 1 and Supplemental Figure 6D online). These results suggest that elevated accumulation of the *CBF2* gene in *rcf1-1* is not due to the increased activity of *CAMTA1*. Together, these data indicate that RCF1 plays an important role in gene regulation under both normal and cold stress conditions.

RCF1 Encodes a Cold-Inducible DEAD Box RNA Helicase

The *rcf1-1* phenotypes indicate that RCF1 has an essential role in chilling and freezing tolerance and in the regulation of cold-responsive gene expression. We used a map-based cloning strategy to identify the *RCF1* gene (Figure 3A). A segregating F2 population was generated from a cross between *rcf1-1* (in the Columbia background) and the wild-type Landsberg *erecta*. A total of 1414 homozygous *rcf1-1* mutant plants were selected from the F2 population, and genomic DNA was extracted from each plant for genetic mapping. The *RCF1* locus is in the lower arm of chromosome 1 between BACs T20H2 and T22I11. The *RCF1* locus was narrowed to BAC clone F9H16, and the candidate genes were sequenced. A single-nucleotide mutation from G to A at position 2650 from the transcription start site was found in *At1g20920* of the *rcf1-1* mutant. This mutation would change the amino acid Gly to Arg at position 808 of the decoded At1g20920 polypeptide. The *At1g20920* gene encodes a putative DEAD box RNA helicase. We confirmed the identity of *RCF1* with a gene complementation test using the wild-type *RCF1* gene, including its own promoter and coding sequences (Figure 3B). We made an RCF1-GFP (for green fluorescent protein) fusion protein in transgenic tobacco (*Nicotiana benthamiana*) and *Arabidopsis* plants to determine the RCF1 subcellular localization, and confocal microscopy indicated that RCF1 is predominantly localized in the nucleus (Figure 3C; see Supplemental Figure 6E online). The RCF1-GFP fusion protein is able to fully restore the *rcf1-1* mutant phenotype (see Supplemental Figure 6F online), indicating that the RCF1-GFP fusion protein is functional in planta. The expression of *RCF1* appears to be upregulated by cold stress and reaches its peak level after 12 h of cold treatment (Figure 3D). qRT-PCR analysis indicated that *rcf1-2* and *rcf1-3* are null alleles of *RCF1* and that the *rcf1-2* and *rcf1-3* mutations have the same effect as the *rcf1-1* mutation on the expression of *CBF* genes (Figure 3E; see Supplemental Figure 6G online).

RNA helicases are a class of enzymes that use energy derived from the hydrolysis of ATP to unwind double-stranded RNAs (de la Cruz et al., 1999). We produced recombinant RCF1 protein and tested its RNA helicase activity by measuring its ATPase activity. RCF1 indeed exhibits RNA-dependent ATPase activity

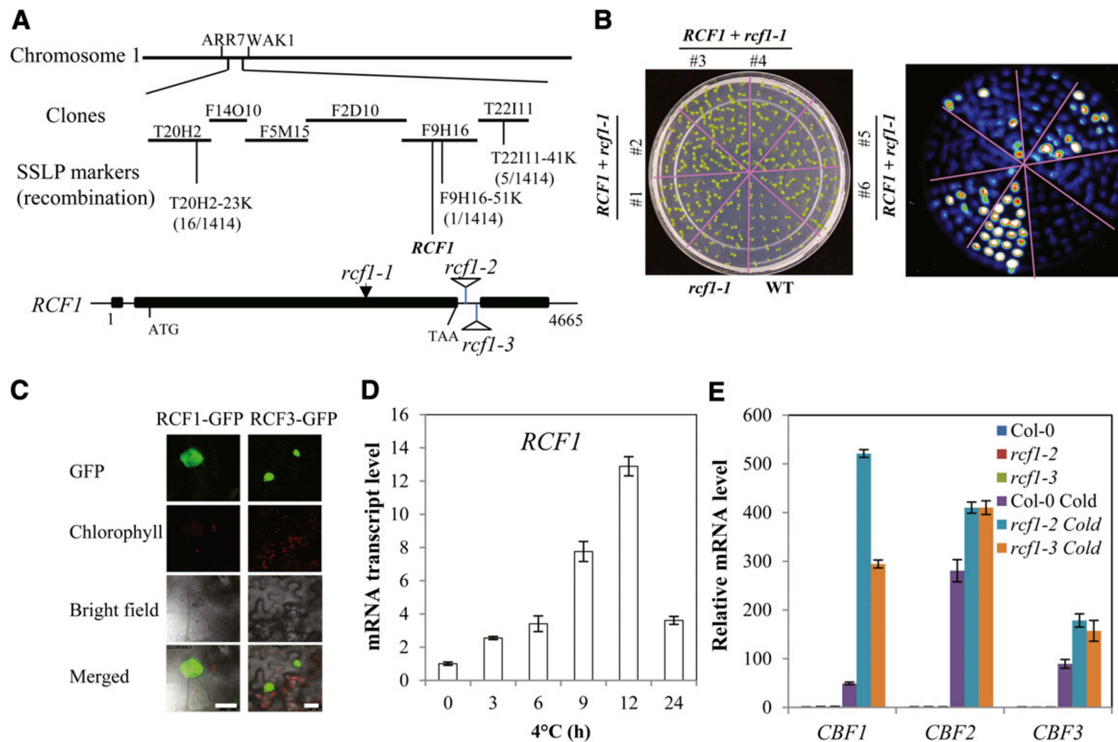

Figure 3. Molecular Cloning of *RCF1*.

(A) Positional cloning of *RCF1*. Numbers of recombination are from 1414 F2 progeny seedlings that are homozygous for *rcf1-1* phenotypes. The *rcf1-1* mutation is caused by a single-nucleotide substitution (from G to A at position 2650, relative to transcription start site), and this mutation changes amino acid Gly-808 to Arg. Structure of the *RCF1* gene and positions of *rcf1-1*, *rcf1-2*, and *rcf1-3* mutations are indicated. Filled boxes indicate exons, and lines between boxes indicate introns.

(B) Molecular complementation of the *rcf1-1* mutant by the wild-type (WT) *RCF1* gene. Shown are seedlings on an MS agar plate (left) and the corresponding luminescence image after 4°C treatment for 24 h (right).

(C) RCF1-GFP is localized in the nucleus of tobacco leaf epidermal cells. RCF3-GFP fusion protein was used as a positive control (Guan et al., 2012). Bars = 25 μm.

(D) Time-course expression of *RCF1* in 14-d-old wild-type seedlings.

(E) Transcript levels of *CBF1*, *CBF2*, and *CBF3* in Col-0, *rcf1-2*, and *rcf1-3* seedlings subjected to 0 or 12 h at 4°C. Error bars in **(D)** and **(E)** indicate the SD (n = 4).

(see Supplemental Figures 7A and 7B online). When the DEAD box domain (core amino acid sequence Asp-Glu-Ala-Asp [D-E-A-D]) is altered to DAAD, the RCF1 ATPase activity is abolished (see Supplemental Figure 7B online). We also found that *RCF1* carrying this DAAD mutation under the control of the *RCF1* native promoter failed to complement the *rcf1-1* mutant (see Supplemental Figure 7C online), indicating that the DEAD box domain of RCF1 is critical for its function in planta.

RCF1 Is Not Involved in mRNA Export

The DEAD box RNA helicase LOS4 was previously identified through a genetic screen of deregulated expression of *RD29A*: *LUC* (Gong et al., 2005). LOS4 is localized in the cytoplasm in a nuclear rim–enriched pattern and is important for mRNA export at cold temperatures (Gong et al., 2005). We used the poly(A) in situ hybridization assay to determine whether RCF1 is also involved in mRNA export. As shown in Supplemental Figure 7D online, the different responses of *los4-1* versus C24 (background of *los4-1*) to the presence and absence of cold stress suggest that our experimental conditions were similar to those reported by Gong et al. (2005), but differences were not detected in the responses of the wild type versus *rcf1-1*. These data indicate that RCF1 is not involved in mRNA export.

RCF1 Is Required for Proper Splicing of Pre-mRNAs for Cold-Responsive Genes Including Positive and Negative Regulators of *CBF*s and for Cold Tolerance

Database searches revealed that *RCF1* coexpresses with a pre-mRNA splicing factor, *STA1*, and a U5 small nucleoprotein helicase, *Brr2b*. We isolated a knockdown mutant of *Brr2b* (see Supplemental Figure 8A online). We found that, like RCF1, STA1 and Brr2b also negatively regulate the expression of *CBF2* (see Supplemental Figure 8B online). Double mutants of *rcf1-1 sta1-2* and *rcf1-1 brr2b* did not show any additive effect on *CBF2* expression (see Supplemental Figure 8B online), suggesting that RCF1, STA1, and Brr2b function in a common pathway to

regulate*CBF2* gene expression. We observed that *Brr2b* is cold inducible (see Supplemental Figure 8C online). Plants of *sta1* and *brr2b* mutants were hypersensitive to chilling stress, as indicated by reduced hypocotyl growth (see Supplemental Figures 8D to 8G online; Lee et al., 2006). Both *sta1-2* and *brr2b* mutant plants were more sensitive to freezing stress than wild-type plants before and after cold acclimation, as determined by an electrolyte leakage assay that indicated increased damage in the membranes of the mutant plants (see Supplemental Figure 8H online). Together, these data suggest that, like RCF1, STA1 and Brr2b are positive regulators of cold tolerance.

Because database searches suggested that RCF1, STA1, and Brr2b are part of a spliceosome containing more than 100 proteins (The Arabidopsis Information Resource) and because STA1 is involved in pre-mRNA splicing (Lee et al., 2006), we determined the potential role of RCF1 in pre-mRNA splicing. To identify candidate genes whose splicing events may be affected by the *rcf1-1* mutation, we performed full-genome tiling arrays with Affymetrix *Arabidopsis* tiling array GeneChips (1.0R) with wild-type and *rcf1-1* plants that had been treated at 4°C for 0 or 12 h. Statistical analysis of the 1.0R tiling array data detected intron retention events (false discovery rate < 0.05) for 204 unique genes in the *rcf1-1* mutant plants subjected to cold treatment for 12 h, which is when *RCF1* reaches its peak expression level (see Supplemental Data Set 3A online). These mis-spliced genes in *rcf1-1* encode proteins with diverse functions in many biological processes, and the predicted roles of more than one-third (77 of 204) of these genes involve responses to abiotic or biotic stresses (see Supplemental Data Set 3A online). We designed primers and probes unique to the introns of genes that are retained in the *rcf1-1* mutant, and we performed RT-PCR and RNA gel blot hybridization analyses to validate the 1.0R tiling array data. Six genes that displayed mis-spliced transcripts in *rcf1-1* as revealed in the 1.0R tiling array experiments with cold-treated plants were selected for validation: *At5g37260*, *At5g54100*, *At5g24470*, *At3g05840*, *At5g25350*, and *At1g27910*. These genes encode MYB family transcription factor CIRCADIAN1 (CIR1), SPFH/PHB DOMAIN-CONTAINING MEMBRANE-ASSOCIATED PROTEIN (SPFH), PSEUDO-RESPONSE REGULATOR5 (PRR5), a SHAGGY-LIKE SERINE/THREONINE KINASE12 (SK12), EIN3 BINDING F BOX PROTEIN2 (EBF2), and PLANT U-BOX45 (PUB45), respectively. Transcripts of the six genes were found to contain at least one intron in the *rcf1-1* mutant under cold treatment (Figures 4A to 4C; see Supplemental Figures 9A and 9B online). The retained introns were not detected in *rcf1-1* plants without cold treatment (Figures 4B to 4D; see Supplemental Figures 9B and 9C online). The *sta1-2* and *brr2b* mutations do not seem to suppress the mis-splicing effect of *rcf1-1* on these six loci (Figure 4A; see Supplemental Figure 9A online), suggesting that RCF1 may function in a different pathway than STA1 and Brr2b for pre-mRNA splicing under cold stress.

Statistical analysis of the 1.0R tiling array data generated with RNA samples extracted from wild-type and *rcf1-1* plants grown under normal conditions did not detect any genes with significant intron retention events (false discovery rate < 0.05) in *rcf1-1* (Gene Expression Omnibus [GEO] accession number GSE41377). Although the 1.0R tiling array data produced from the unstressed wild-type and *rcf1-1* plants did not pass the thresholds in our statistical analysis, we were able to confirm the intron retention events through RT-PCR analysis for the following six genes: *At3g21350*, *At1g65660*, *At4g25080*, *At3g52115*, *At1g21590*, and *At1g73670*. *At3g21350* encodes MEDIATOR6 (MED6), which is an important component of the mediator complex in the regulation of RNA polymerase II transcription activity. *At1g65660* encodes SWELLMAP1 (SMP1), which is a CCHC zinc finger protein that may function as a step II splicing factor. *At4g25080* encodes a protein with methyltransferase activity responsible for the methylation of magnesium protoporphyrin IX (CHLM). *At3g52115* encodes GAMMA RESPONSE GENE1 (GR1), which is induced in response to ionizing radiation and may be involved in DNA damage-induced growth arrest. *At1g21590* encodes a protein kinase with an adenine nucleotide α-hydrolase–like domain. *At1g73670* encodes MAP KINASE15 (MPK15) (see Supplemental Data Set 3B and Supplemental Figure 10 online; Figure 4D). These encoded proteins have diverse functions in cellular processes, including signal transduction (MPK15), gene regulation (SMP1 and MED6), and stress responses (GR1 and At1g21590) (see Supplemental Data Set 3B online).

Searches of the publically available microarray databases revealed that many of the mis-spliced genes in *rcf1-1* under cold stress are cold responsive in wild-type plants, suggesting that they play a role in the cold stress tolerance pathway (see Supplemental Figure 9D online). Indeed, *CIR1*, *SPFH*, *SK12*, and *PRR5* are cold inducible (Figure 5A). To investigate possible functions of these four genes in cold stress responses, we isolated their T-DNA knockouts (see Supplemental Figures 11A to 11D online). As shown in Figures 5B to 5D, expression of *CBFs* and *RD29A* is substantially reduced in *cir1* and *spfh* plants under cold stress, and these mutant plants are hypersensitive to freezing stress before and after cold acclimation. Furthermore, transgenic plants that overexpress *CIR1* (*CIR1 OE*) and *SPFH* (*SPFH OE*) display increased expression of *CBF* genes and increased tolerance to freezing stress before and after cold acclimation (Figures 5E and 5F; see Supplemental Figure 11E online). These results indicate that CIR1 and SPFH are positive regulators for cold-responsive gene expression and cold tolerance. By contrast, *sk12* and *prr5* plants displayed elevated expression of *CBFs* and *RD29A* under cold and increased tolerance to freezing temperatures (Figures 5B to 5D). In addition, transgenic plants that overexpress *SK12* (*SK12 OE*) and *PRR5* (*PRR5 OE*) showed reduced expression of *CBF* genes and decreased tolerance to freezing stress (Figures 5E and 5F; see Supplemental Figure 11F online). These results suggest that *SK12* and *PRR5* are negative regulators of cold-responsive gene expression and cold tolerance.

Overexpression of *RCF1* in *Arabidopsis* Increases Tolerance to Chilling and Freezing Stresses

Because RCF1 is required for cold stress tolerance and is important for cold-responsive gene regulation, we investigated whether overproduction of RCF1 would improve the performance of plants under cold stress. We generated transgenic *Arabidopsis* plants that overexpress *RCF1* under the control of the 35S promoter, and we selected two independent *RCF1* overexpression lines (Figure 6A). The *RCF1* overexpression lines developed longer hypocotyls than the wild type under chilling

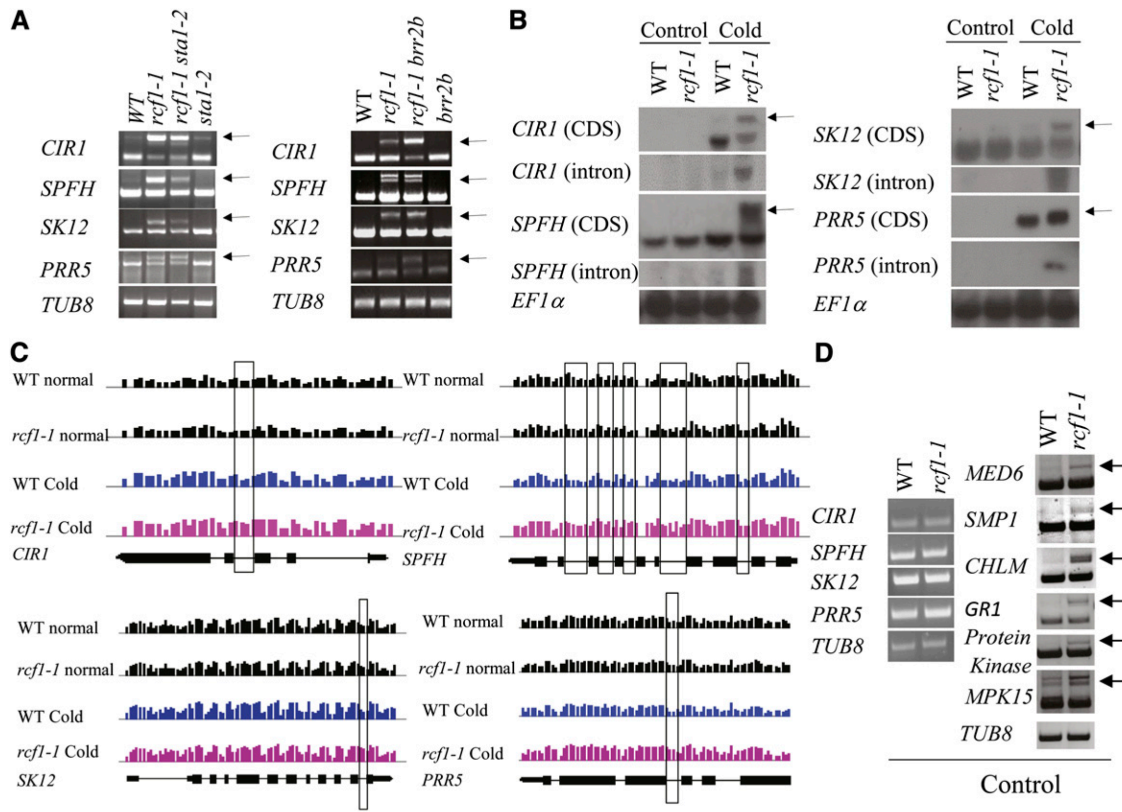

Figure 4. RCF1 Functions in Pre-mRNA Splicing under Cold Treatment.

(A) Mis-spliced transcripts of *CIR1*, *SPFH*, *SK12*, and *PRR5* in *rcf1-1*, *rcf1-1 sta1-2*, and *rcf1-1 brr2b*, but not in *sta1-2* or *brr2b*, under cold stress (4°C for 12 h) as determined by RT-PCR analysis. WT, the wild type.

(B) RNA gel blot hybridization analysis of *CIR1*, *SPFH*, *SK12*, and *PRR5* genes in *rcf1-1*. Membranes were also hybridized with introns specific to *CIR1*, *SPFH*, *SK12*, and *PRR5*.

(C) Visualization of intron retention of *CIR1*, *SPFH*, *SK12*, and *PRR5* in *rcf1-1* with the integrated genome browser. Vertical bars represent averaged $\log_2$ expression values of unique probes in the gene region; introns that were retained in *rcf1-1* under cold treatment are indicated with black boxes; gene structures on the bottom of each panel are based on TAIR10 annotation.

(D) Transcripts of genes tested in **(A)** and *MED6* (*At3g21350*), *SMP1* (*At1g65660*), *CHLM* (*At4g25080*), *GR1* (*At3g52115*), *protein kinase* (*At1g21590*), and *MPK15* (*At1g73670*) under unstressed condition. *TUB8* and *EF1α* were used as loading controls.

Arrows in **(A)**, **(B)**, and **(D)** indicate intron-retained transcripts. The experiments in (except in **[C]**) were repeated at least three times with similar results, and data shown are from one experimental repetition.

[See online article for color version of this figure.]

stress, indicating an increased resistance to chilling stress (Figures 6B and 6C). Expression of *CBF* genes is repressed in the *RCF1* overexpression lines (Figure 6D). These results further support our conclusion that RCF1 is a negative regulator for these cold-responsive transcriptional activators. In an electrolyte leakage assay, the RCF1 overexpression lines displayed significantly increased tolerance to freezing temperature with or without cold acclimation (Figure 6E). Together, these results further support our conclusion that RCF1 is required for cold tolerance in plants.

DISCUSSION

We identified the cold-inducible DEAD box–containing RNA helicase RCF1 through a forward genetic screen for genes critical for cold tolerance. We confirmed that recombinant RCF1 protein produced in *Escherichia coli* has RNA helicase activity and that changing the functional DEAD box to DAAD abolishes its helicase activity (see Supplemental Figure 7 online). Similarly, the *RCF1* gene carrying the DAAD mutation in the DEAD box failed to complement the *rcf1-1* mutant, suggesting that the DEAD domain or the RNA helicase activity of RCF1 is essential for its biological function.

The RCF1 protein differs from a previously identified DEAD box RNA helicase, LOS4, in subcellular localization and function. Although both proteins are localized in the nucleus, LOS4 is enriched in the nuclear rims (Gong et al., 2005), but RCF1 is not (Figure 3C; see Supplemental Figure 6E online). The subcellular localization of LOS4 is correlated with its important role in mRNA export at cold temperatures (Gong et al., 2005), and the different location for RCF1 indicates that it is probably not involved in

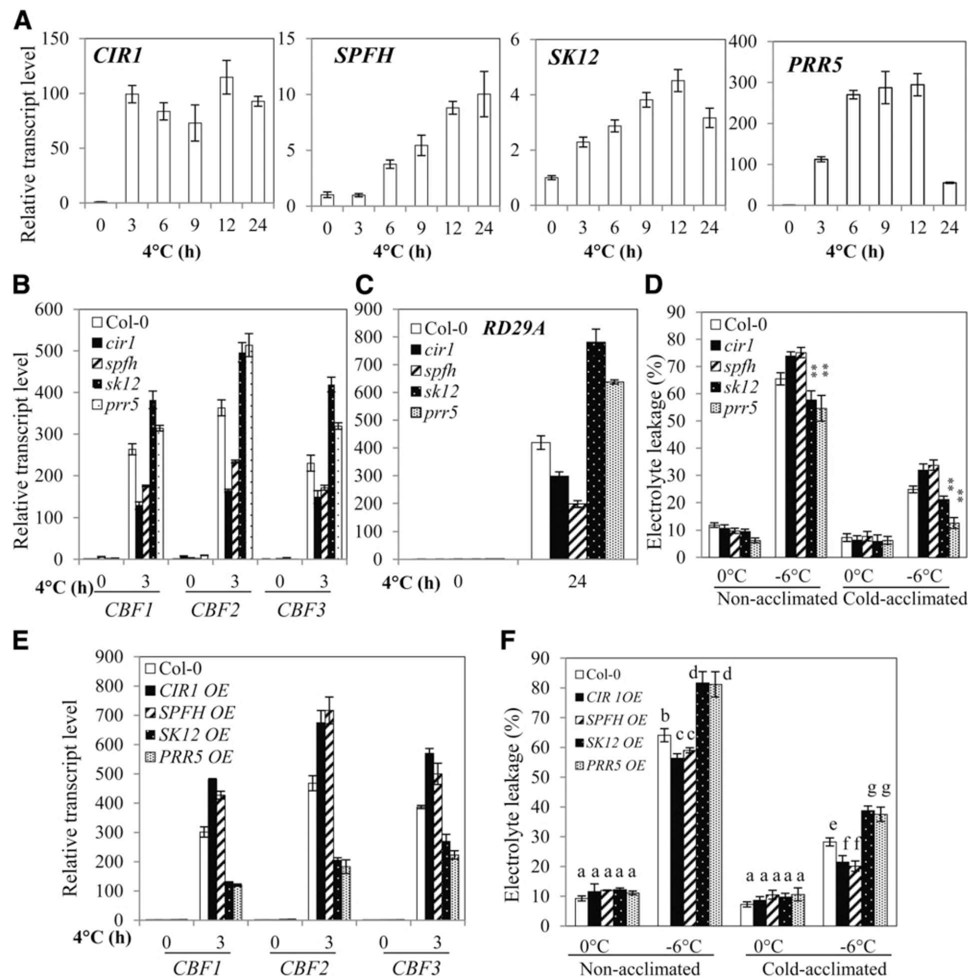

Figure 5. *CIR1*, *SPFH*, *SK12*, and *PRR5* Regulate Expression of *CBF* Genes and Cold Tolerance.

(A) *CIR1*, *SPFH*, *SK12*, and *PRR5* are cold inducible in the wild type.

(B) and **(C)** Relative transcript levels of *CBF1*, *CBF2*, *CBF3*, and *RD29A* in *cir1*, *spfh*, *sk12*, and *prr5* mutant plants.

(D) Freezing tolerance of *cir1*, *spfh*, *atsk12*, and *prr5* mutant plants determined by electrolyte leakage assays. **P < 0.01, as determined by Student's *t* test.

(E) Relative transcript levels of *CBF1*, *CBF2*, and *CBF3* in overexpression plants of *CIR1* (*CIR1 OE*), *SPFH* (*SPFH OE*), At-*SK12* (*SK12 OE*), and *PRR5* (*PRR5 OE*).

(F) Freezing tolerance of *CIR1 OE*, *SPFH OE*, *AtSK12 OE*, and *PRR5 OE* plants. Cold acclimation in **(D)** and **(F)** was achieved by incubating plants at 4°C for 1 week. Data in **(E)** and **(F)** are from one representative transgenic line of two independent transgenic lines of *CIR1 OE*, *SPFH OE*, At-*SK12 OE*, and *PRR5 OE* plants (see Supplemental Figures 11E and 11F online).

Error bars indicate the SD (*n* = 4 in **[B]**, **[C]**, and **[E]**; *n* = 12 to 15 in **[D]** and **[F]**). One-way ANOVA (Tukey-Kramer test) was performed for data in **(F)**, and statistically significant differences are indicated by different lowercase letters (P < 0.01). Experiments in were repeated at least four times with similar results, and values shown are from one experimental repetition.

mRNA export. A role of RCF1 in mRNA export was ruled out by the poly(A) in situ hybridization assays (see Supplemental Figure 7D online).

DEAD box RNA helicases contribute to all aspects of RNA metabolism, including nuclear gene transcription, pre-mRNA splicing, nucleo-cytoplasmic transport, and gene expression (Rocak and Linder, 2004; Cordin et al., 2006). In this study, three lines of evidence demonstrate the role of RCF1 in pre-mRNA splicing. First, RCF1 coexpresses with another RNA helicase, Brr2b, and a pre-mRNA splicing factor, STA1. Second, database searches indicated that RCF1, STA1, and Brr2b are part of a spliceosome composed of over 100 proteins. Although there is no experimental evidence that Brr2b is involved in pre-mRNA splicing in *Arabidopsis*, a Brr2b homolog in yeast is involved in pre-mRNA splicing (Hahn et al., 2012). We therefore reasoned that RCF1 may function in some aspects of splicing. Third, full-genome tiling array analyses indicated that RCF1 regulates pre-mRNA splicing of six genes without cold stress and 204 unique genes under cold stress (see Supplemental Data Set 3 online). Because the mis-spliced genes in *rcf1-1* under cold stress are

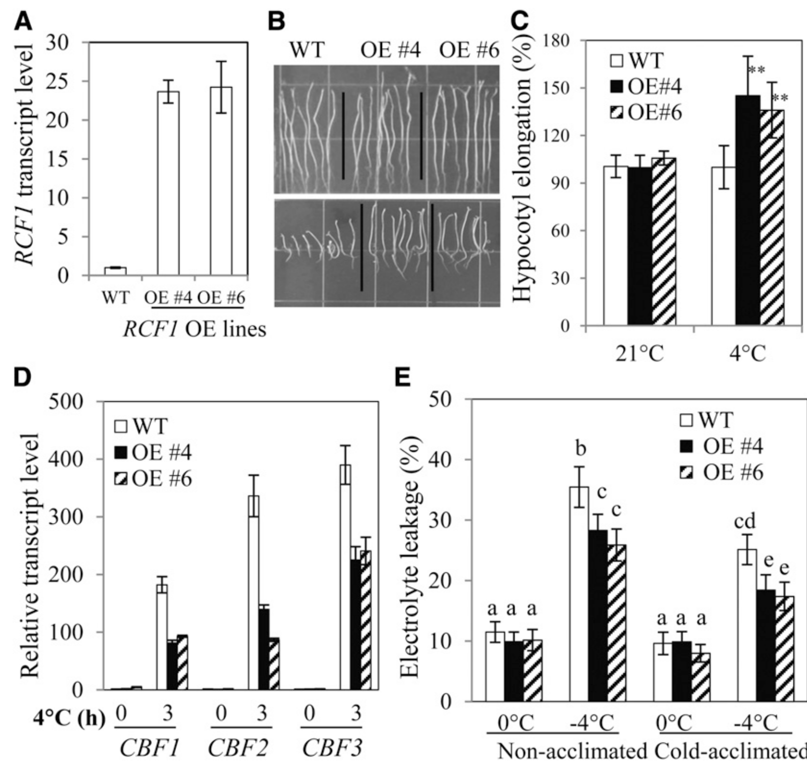

Figure 6. Overexpression of *RCF1* Increases Cold Stress Tolerance.

(A) *RCF1* expression levels in the wild type (WT) and two transgenic lines overexpressing *RCF1* (OE).

(B) Chilling stress tolerance of wild-type and *RCF1* OE plants. Top panel, 21°C for 13 d; bottom panel, 4°C in the dark for 32 d.

(C) Quantification of hypocotyl elongation of plants shown in **(B)**. Hypocotyl elongation of the wild type (at 21°C or 4°C) was considered to be 100%. **P < 0.01, as determined by Student's *t* test.

(D) Expression of *CBF1*, *CBF2*, and *CBF3* in wild-type and *RCF1* OE plants subjected to 0 or 3 h at 4°C.

(E) Freezing tolerance of *RCF1* OE plants determined by electrolyte leakage assays.

Cold acclimated (4°C for 1 week). Error bars indicate the SD (*n* = 4 in **[A]** and **[D]**; *n* = 20 in **[C]**; *n* = 12 in **[E]**). One-way ANOVA (Tukey-Kramer test) was performed for data in **(E)**, and statistically significant differences are indicated by different lowercase letters (P < 0.005). Experiments in were repeated at least three times with similar results, and values shown are from one experimental repetition.

not affected in *sta1-2* or *brr2b* mutants, RCF1 seems to function in the pre-mRNA splicing pathway independently of STA1 and Brr2b. Based on our results, we believe that RCF1, Brr2b, and STA1 may be components of different pre-mRNA splicing complexes that regulate control the pre-mRNA splicing of different genes. That our 1.0R tiling array experiments detected only a relatively small number of mis-spliced genes in *rcf1-1* under normal growth condition (Figure 4D; see Supplemental Figure 10 online; GEO accession number GSE41377) may be explained by the fact that the 1.0R tiling arrays used in this study may be insufficiently sensitive to detect all mis-splicing events.

Our data indicate that *rcf1-1* mutant plants are defective in basal freezing tolerance (Figures 1E to 1G) and that the reduced basal freezing tolerance may be due to mis-splicing of the six genes in *rcf1-1* under normal conditions (Figure 4D). Two of the six mis-spliced genes (ATGR1 and a protein kinase) in *rcf1-1* under normal conditions have predicted roles in stress responses (see Supplemental Data Set 3B online). Mis-splicing of these two genes in *rcf1-1* under normal conditions may reduce the ability of *rcf1-1* to cope with freezing temperatures without cold acclimation. Two additional genes whose transcripts are mis-spliced in *rcf1-1* under normal conditions (*SMP1* and *MED6*) are involved in gene regulation. *SMP1* encodes a CCHC zinc finger protein with step II splicing factor activity. The spliceosome catalyzes pre-mRNA splicing in two steps. After catalytic step I, a major remodeling of the spliceosome occurs to establish the active site for step II. Step II splicing factors are responsible for the correct selection of 3′ splicing sites (Chua and Reed, 1999). SMP1 is functionally redundant with SMP2. One of the functional targets of SMP1 and SMP2 is the transcript of *STRUWWELPETER* that resembles the subunits of the mediator complex required for transcriptional activation (Clay and Nelson, 2005). MED6 is one of the mediators of RNA polymerase II (Pol II). The mediator of Pol II is required for diverse aspects of the transcription process, including activation, repression, basal transcription, and phosphorylation of the C-terminal domain of the largest subunit of Pol II (Kim et al., 1994; Björklund and Kim, 1996). MED6 homologs in *Drosophila melanogaster* and yeast play important roles as transcriptional coactivators (Lee and Kim, 1998; Gim et al., 2001). The MED6 in *Arabidopsis* may

function similarly to its homologs in yeast and *Drosophila*. Furthermore, splicing of *MPK15* is defective in *rcf1-1* under normal conditions, and MPK15 is likely involved in the activation of gene expression. As mentioned above, MPK15, SMP1, and MED6 are involved in gene regulation, and mis-splicing of these three genes in *rcf1-1* may alter expression of genes, for example, those genes that are differentially expressed in *rcf1-1* under nonstressed conditions (see Supplemental Table 2 online). The altered gene expression in *rcf1-1* may contribute to reduced basal freezing tolerance.

Although *rcf1-1* plants should theoretically display enhanced freezing tolerance when transcripts of cold-responsive genes accumulate to a higher level, the opposite phenotype was observed (Figures 1 and 2). Our results show that RCF1 is required for tolerance to chilling and freezing stresses in plants because *rcf1-1* mutant plants are hypersensitive to cold stress, and overexpression of *RCF1* increases cold tolerance (Figures 1 and 6). These results suggest that an essential regulator(s) of cold tolerance is defective in *rcf1-1*. *CIR1* and *SPFH* are two good candidates for such essential regulators of cold tolerance, and transcripts of these two genes were mis-spliced in *rcf1-1* under cold stress (Figure 4). CIR1 is involved in circadian-regulated developmental processes, and circadian rhythmic expression of *CIR1* is regulated by the central oscillators CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two MYB transcription factors that have partially redundant functions (Schaffer et al., 1998; Wang and Tobin, 1998; Mizoguchi et al., 2002; Zhang et al., 2007). The functions of CCA1 and LHY in the regulation of *CBF* expression have been reported (Dong et al., 2011), but the function of CIR1 in cold stress response has not been determined. SPFH is one of the SPFH/PHB (stomatin-prohibtin-flotillin-HflC/K) domain-containing superfamily proteins, and members of the SPFH protein superfamily are generally associated with plasma or mitochondrial membranes and are involved in many cellular processes, including protein turnover or oligomerization, cell proliferation, and ion channel regulation (Tavernarakis et al., 1999; Nadimpalli et al., 2000; Rivera-Milla et al., 2006). According to our investigation of the loss-of-function and gain-of-function of the *CIR1* and *SPFH* genes, CIR1 and SPFH positively regulate expression of cold-responsive genes, including *CBFs*, and are required for cold tolerance (Figures 5B to 5F). *CIR1* and *SPFH* appear to function in the cold stress tolerance pathway in a *CBF*-dependent manner. In addition, the list of RCF1 target genes as revealed by the ATH1 microarray and 1.0R tiling array analyses might include other positive and negative regulators that are essential for cold stress tolerance (for example, factors functioning in CBF-independent pathways). The molecular function of these positive and negative regulators for cold tolerance requires further study, but indications about these regulators were provided by the gene expression data generated in our microarray and tiling array analyses. Although the differentially expressed genes in *rcf1-1* with or without cold stress encode proteins that are involved in diverse biological processes, many of the upregulated genes and some of the downregulated genes in *rcf1-1* encode proteins that are predicted to function in the biotic or abiotic stress response pathways (see Supplemental Table 2 and Supplemental Data Sets 1 and 2 online). Gene expression data in *rcf1-1* with and without cold treatment were quite different from publically available gene expression data from wild-type plants (see Supplemental Figures 1 to 5 online). In the latter case, genes are normally upregulated in wild-type plants after cold stress, but these genes failed to increase in *rcf1-1* in response to cold (see Supplemental Figures 3 and 5 online). Similarly, genes that are repressed by cold stress in the wild type are turned on in *rcf1-1* (see Supplemental Figures 1A, 2, and 4 online). Aberrant expression of these genes in *rcf1-1* under cold stress potentially compromises the mutant's ability to cope with freezing temperatures. Furthermore, many of the mis-spliced genes in *rcf1-1* revealed by the 1.0R tiling array analyses are responsive to cold stress in wild-type plants, suggesting that they are involved in cold stress responses (see Supplemental Figure 9D online). The mis-splicing and resulting disruption of functions of some of these genes in *rcf1-1* plants may contribute to the increased cold sensitivity of *rcf1-1*.

The increased accumulation of *CBF* genes and their downstream targets in *rcf1-1* may be explained by the action of RCF1 target genes as revealed in the 1.0R tilling array experiments. PRR5 and SK12 appear to be negative regulators of *CBFs* and *RD29A* (Figures 5B and 5C). *PRR5* is strongly induced by cold, and *SK12* is moderately upregulated by cold (Figure 5A). PRR5 is a negative regulator of *CBF* genes and is partially redundant with PRR7 and PRR9 (Nakamichi et al., 2009). The null allele of *prr5* used in this study (see Supplemental Figure 11D online) differed from those reported previously (Nakamichi et al., 2009), and this single mutation was sufficient to display phenotypes such as altered expression of *CBF* genes and sensitivity to freezing temperatures. Mis-spliced transcripts of *PRR5* and *SK12* in *rcf1-1* presumably disrupt the normal function of these two genes, leading to increased induction of *CBF* genes in *rcf1-1* in a similar way to that observed in the *prr5* and *sk12* mutant plants. Overexpression of *PRR5* or *SK12* results in reduced expression of *CBF* genes and reduced freezing tolerance (Figures 5E and 5F), confirming that PRR5 and SK12 are negative regulators of cold-responsive gene expression and cold tolerance.

There are additional examples of mutants that are more sensitive to cold stress than the wild type even though they accumulate higher levels of cold-responsive genes. The *sta1-1*, *sta1-2*, and *brr2b* mutant plants are hypersensitive to chilling and freezing stresses, although the *CBF2* gene is expressed at a higher level in these three mutants (see Supplemental Figure 8 online). Thus, STA1 and Brr2b are required for cold tolerance. Because the cold response of *rcf1-1* is similar to that of *sta1* and *brr2b*, it is possible that RCF1, STA1, and Brr2b share a common subset of target genes critical for cold tolerance. Identification of these common target genes regulated by RCF1, STA1, and Brr2b requires further investigation. Nevertheless, STA1 and Brr2b are two examples (in addition to RCF1) of important proteins that are required for cold tolerance and that act as negative regulators of *CBF* gene expression. Increased expression of *CBF2* in *sta1-2* and *brr2b* can be a compensatory response to an increased sensitivity to cold stress, as was the case with *sta1-2* and *brr2b*. The overaccumulated transcripts of *CBF* genes in *rcf1-1* may be a compensatory response to the increased sensitivity of *rcf1-1* to cold stress. The underlying mechanism for this type of compensatory response has been

a mystery. Our results show that the defective splicing of *SK12* and *PRR5* explains at least part of the compensatory response in the *rcf1-1* mutant. We have observed a similar phenomenon in *hos9-1* and *hos15* mutants, which accumulate higher levels of some cold-responsive genes but are hypersensitive to cold stress (Zhu et al., 2004, 2008). Unlike *rcf1-1*, however, *hos9-1* and *hos15* mutations do not affect cold induction of *CBF* genes.

The importance of RCF1 protein in cold stress responses is further supported by the consequences of *RCF1* overexpression in *Arabidopsis*. Overexpression of *RCF1* enhances the tolerance to chilling and freezing stresses (Figure 6), indicating that RCF1 and the splicing regulated by it are essential for cold tolerance. Furthermore, expression of *CBF* genes was downregulated in *RCF1* overexpression lines, confirming that RCF1 is indeed a negative regulator of *CBF* genes. When *RCF1* is overexpressed, positive regulators for cold tolerance are presumably produced at a higher level, and negative regulators are presumably suppressed to improve performance under cold stress. Although determining precisely how RCF1 functions in cold tolerance will require further investigation, our forward genetic analysis has demonstrated that by maintaining splicing of pre-mRNAs under cold conditions, RCF1 is a crucial regulator of cold tolerance in plants.

METHODS

Plant Materials and Growth Conditions

A firefly *LUC* reporter gene driven by the cold stress-responsive *CBF2* promoter (−1500 to −1 bp upstream of the transcription start site) was introduced into *Arabidopsis thaliana* plants in the Columbia *glabrous1* background. Seeds from one homozygous line expressing a single functional copy of the *CBF2:LUC* gene (referred to as the wild type) were mutagenized with EMS. The *rcf1-1* mutant with altered *CBF2:LUC* gene expression was identified from M2 seedlings using a charge-coupled device camera imaging system (Ishitani et al., 1997).

Seeds of the following T-DNA insertion mutants were obtained from the ABRC: *rcf1-2* (SALK_138032), *rcf1-3* (SALK_062599), *sta1-2* (SAIL_262-C08), *brr2b* (SALK_048780), *prr5* (SALK_135000), *sk12* (CS856017), *cir1* (SALK_051843), and *spfh* (SALK_090074). The *sta1-1* mutant was described previously (Lee et al., 2006). *Arabidopsis* seedlings on Murashige and Skoog (MS) medium agar plates (1× MS salts, 2% Suc, and 0.6% agar, pH 5.7) were routinely grown under cool, white light (~120 μmol m^{-2} s^{-1}) at 21 ± 1°C with a 16-h-light/8-h-dark photoperiod. Soil-grown plants were kept under cool, white light (~100 μmol m^{-2} s^{-1}) with a 16-h-light/8-h-dark photoperiod at 21 ± 1°C and with a 1:1 ratio of Metro Mix 360 and LC1 potting soil (Sun Gro Horticulture).

Chilling and Freezing Tolerance Assays

For chilling tolerance assays, seeds of relevant genotypes were sown side-by-side on agar plates containing MS medium. These agar plates were then wrapped with aluminum foil and kept vertically at 4°C or 21°C in growth chambers for the desired time. Reduction in hypocotyl elongation at 4°C relative to that of the wild type at 21°C was used as an indicator of sensitivity to chilling stress.

To evaluate freezing tolerance using electrolyte leakage assays, 3-week-old wild-type and mutant or transgenic plants were grown in soil at room temperature or at 4°C under a long-day photoperiod (16-h-light/8-h-dark) for 1 week. Fully developed rosette leaves were used for electrolyte leakage measurements as described (Sukumaran and Weiser, 1972; Ristic and Ashworth, 1993; Zhu et al., 2004). Whole-plant freezing tests were as described (Xiong et al., 2001) with modifications. Wild-type and *rcf1-1* plants were grown in soil under a long-day photoperiod (16-h-light/8-h-dark) in a growth chamber for 3 weeks at 21°C and then at 4°C for 1 week for cold acclimation. The plants were then placed in a low-temperature chamber with the following freezing temperature regimen: from 4 to −1°C in 30 min, then hold at −1°C for 1 h (to initiate nucleation); then successive 2°C decreases at 30-min intervals to reach the next temperature (hold at the following temperatures for 3 h: −4, −6, −8, −10, and −12°C). Plant damage was scored 7 d later (Xiong et al., 2001).

Genetic Mapping and Complementation

The *rcf1-1* mutant was crossed with the Landsberg *erecta* accession, and 1414 mutant plants were chosen from the F2 generation based on altered *CBF2:LUC* phenotype. Simple sequence length polymorphism markers were designed according to the information in the Cereon *Arabidopsis* Polymorphism Collection and were used to analyze recombination events (Jander et al., 2002). Initial mapping revealed that the *rcf1-1* mutation is located on the upper arm of chromosome 1 between T20H2 and T22I11. Fine mapping within this chromosomal interval narrowed the *RCF1* locus to the BAC clone F9H16. All candidate genes in this BAC were sequenced from the *rcf1-1* mutant and compared with those in GenBank to find the *rcf1-1* mutation.

For complementation of the *rcf1-1* mutant, a 6975-bp genomic fragment of *At1g20920* that included 2224 bp upstream of the translation initiation codon and 1251 bp downstream of the translation stop codon was amplified with F9H16 as a template (see Supplemental Data Set 4 online for primer sequences). The amplified fragment was first cloned through Gateway technology (Invitrogen) into the pENTR4 vector, resulting in plasmid pENTR4-RCF1. The pENTR4-RCF1 plasmid was then subjected to site-directed mutagenesis to change the DEAD domain into the DAAD mutant version at the amino acid level with the primer pair listed in Supplemental Data Set 4 online. The wild-type *RCF1* gene and mutated *RCF1* (DEAD to DAAD) were then introduced into pMDC99. The constructs were transferred into *Agrobacterium tumefaciens* (strain GV3101), and *rcf1-1* plants were transformed by the floral dip method (Clough and Bent, 1998).

RCF1 Subcellular Localization and Overexpression of *RCF1*, *PRR5*, *SK12*, *CIR1*, and *SPFH*

The coding region of *RCF1* was amplified by PCR and cloned into the pMDC83 vector to produce the RCF1-GFP fusion protein. This construct (pMDC83-RCF1) was then introduced into *Arabidopsis* wild-type (ecotype Columbia) and *rcf1-1* plants by floral dip transformation with *Agrobacterium* strain GV3101. The pMDC83-RCF1 plasmid was also transformed into *Agrobacterium* strain C58C1 and coinfiltrated with *35S:p19* (p19 is a RNA silencing repressor protein from tomato bushy stunt virus; Voinnet et al., 2003) in *Agrobacterium* strain C58C1 into the 3-week-old leaves of tobacco (*Nicotiana benthamiana*) plants. The infiltrated tobacco plants were allowed to grow for an additional 3 d in a growth chamber under a 16-h-light/8-h-dark photoperiod at 21°C. The subcellular localization of RCF1-GFP protein in tobacco leaves or in root tissues of *Arabidopsis* transgenic plants (T2 generation) was determined with a Leica SP5X confocal microscope (Leica Microsystems). The *rcf1-1* mutant plants transformed with pMDC83-RCF1 in the T2 generation were examined for *CBF2:LUC* expression to determine whether the pMDC83-RCF1 fusion protein is functional in planta. Nuclear localization of pMDC83-RCF3 fusion protein in tobacco leaves was used as a positive control. RCF3 protein was described previously (Guan et al., 2012).

For overexpression of *RCF1*, *PRR5*, *SK12*, *CIR1*, and *SPFH*, coding regions of these genes were amplified by PCR and cloned into the

pMDC32 vector. The resulting plasmids (pMDC32-RCF1, pMDC32-PRR5, pMDC32-SK12, pMDC32-CIR1, and pMDC32-SPFH) were then transferred into *Agrobacterium* (strain GV3101), and wild-type *Arabidopsis* plants (ecotype Columbia) were transformed by the floral dip method. Transgenic plants resistant to hygromycin (50 µg/mL) in the T2 generation were tested for resistance to cold stress and for gene regulation under cold stress.

Microarray Analysis, Tilling Array Analysis, and Real-Time RT-PCR Analysis

Fourteen-day-old seedlings grown on MS medium were used for RNA isolation. Total RNA was extracted from the wild type, different mutants, and/or transgenic plants with Trizol reagent (Invitrogen) and treated with DNase I (New England Biolabs) to remove any genomic DNA contaminants.

For GeneChip *Arabidopsis* Genome (ATH1, Affymetrix) array analysis, total RNA was used to prepare biotin-labeled complementary RNA targets. Microarray analysis was performed at the School of Medicine, University of Maryland at Baltimore, as described (Breitling et al., 2004). Two biological replicates were used for each genotype. The data sets were subjected to the robust multiarray averaging normalization method. The robust multiarray averaging method for computing an expression measure is begun by computing background-corrected perfect match intensities for each perfect match cell on every GeneChip. The normalized data were further analyzed, and P values were generated by the affylmGUI component of Bioconductor in statistics environment R with the default parameters (Irizarry et al., 2003; Gentleman et al., 2004). Genes with statistically significant differences in expression between the *rcf1-1* mutant and the wild type were selected by the RankProd method, which is a nonparametric method for identifying differentially expressed up- or downregulated genes based on the estimated percentage of false discoveries ($P < 0.05$) (Hong et al., 2006). RankProd results were summarized with the script written in PERL.

For GeneChip *Arabidopsis* Tiling (1.0R, Affymetrix) array analysis, total RNA extracted from cold-treated (4°C for 12 h, which is when RCF1 is expressed at its peak level) wild-type and *rcf1-1* plants was used, and labeling, hybridization, and scanning were performed in the Genomics Core of the Institute for Integrative Genome Biology at the University of California at Riverside. Three biological replicates were used for each genotype. We first remapped the tiling array probes to the *Arabidopsis* genome (TAIR10) using SOAP2 (Li et al., 2009) and kept only probes that perfectly matched to a unique position in the genome for subsequent analyses. We then created a custom chip definition file using the probe mapping result and used the aroma.affymetrix framework (Bengtsson et al., 2008) to quantile normalize the raw tiling array data (three biological replicates each for the *rcf1-1* mutant and wild-type control). To identify retained introns, we first calculated the $\log_2$ signal intensity for each annotated intron (TAIR10) based on the trimmed mean of signal intensities from all mapped probes. Introns with fewer than three mapped probes or with low expression ($\log_2$ expression value < 5 in all samples) were not further considered. The SAM algorithm (Tusher et al., 2001) was then used to identify introns with significantly elevated expression in the *rcf1-1* mutant samples relative to the wild type. A false discovery rate of 0.05 was used as the significance cutoff. Intron retention in *rcf1-1* of selected genes under both normal and cold stress conditions was visualized with the integrated genome browser (http://bioviz.org/igb/; Nicol et al., 2009).

For qRT-PCR analysis, 5 µg of total RNA was used to synthesize the first-strand cDNA with the Maxima first-strand cDNA synthesis kit (Fermentas) as described (Guan et al., 2012). Each experiment had three to five biological replicates (four technical replicates for each biological replicate), and each experiment was repeated at least three times. The comparative cycle threshold method was applied, and *TUB8* was used as a reference gene.

RNA Gel Blot Hybridization Analysis

Fourteen-day-old seedlings grown on MS medium and subjected to 0- or 12-h cold treatment at 4°C were used for RNA isolation. Total RNA was extracted with Trizol reagent (Invitrogen) and treated with DNase I (New England Biolabs) for potential genomic DNA contamination. Total RNA (30 µg) was then separated on a formaldehyde-containing agarose (1.2%, [w/v]) gel, transferred to a nylon transfer membrane (GE Healthcare) overnight, and cross-linked. Blots were hybridized overnight (0.5 M phosphate buffer, pH 7.2, 7% [w/v] SDS, 1 mM EDTA, and 2 mM BSA) with ^{32}P-labeled probe at 60°C. Washes (20 min each at 60°C) were performed first in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS, then in 1× SSC and 0.1% SDS, and finally in 0.5× SSC and 0.1% SDS. The *ELONGATION FACTOR1α* (*EF1α*) gene was used as a loading control.

ATPase Activity Assay

A fragment of RCF1 cDNA (corresponding to amino acids 501 to 910 from the N-terminal region, spanning the ATPase motif) was amplified with the primer pair listed in Supplemental Data Set 3 online and cloned into pDEST15 to make a glutathione *S*-transferase–tagged GST-RCF1 fusion protein. The resulting plasmid pDEST15-RCF1 was used as a template to make a mutant version of RCF1 (DEAD domain changed to DAAD) through site-directed mutagenesis (pDEST15-RCF1 [DAAD]). The pDEST15-RCF1 and pDEST15-RCF1 (DAAD) plasmids were transformed into *Escherichia coli.* Rosetta (DE3)pLysS cells (EMD Millipore) and fusion proteins were purified with glutathione *S*-transferase affinity columns (GE Healthcare). The ATPase activities of RCF1 and its mutant version were determined as described by Iost et al. (1999) with minor modifications. This method uses pyruvate kinase and lactate dehydrogenase to link hydrolysis of ATP to oxidation of NADH, which results in a decrease in the absorbance at 338 nm. Briefly, assays were performed at 37°C in a reaction volume of 0.2 mL, in buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 300 μM NADH, 2 mM phosphoenolpyruvate, 73.5 nM RCF1 (or RCF1 [DAAD]), and 3 units/mL of pyruvate kinase and lactate dehydrogenase with or without 50 μg/mL *Arabidopsis* total RNA.

Poly(A) RNA in Situ Hybridization Assay

Poly(A) RNA in situ hybridization was conducted essentially as described by Engler et al. (1994) and Gong et al. (2005). The samples were observed immediately using a Leica SP5X confocal microscope (Leica Microsystems) with a 488-nm excitation laser and a 522/DF35 emission filter. All samples were observed under the same conditions, including the use of the same ×60 objective lens and the same laser strength.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *RCF1* (*At1g20920*), *STA1* (*At4g03430*), *Brr2b* (*At2g42270*), *CBF1* (*At4g25490*), *CBF2* (*At4g25470*), *CBF3* (*At4g25480*), *RD29A* (*At5g52310*), *PRR5* (*At5g24470*), *SK12* (*At3g05840*), *PUB45* (*At1g27910*), *EBF2* (*At5g25350*), *CIR1* (*At5g37260*), *SPFH* (*At5g54100*), *COR15A* (*At2g42540*), *MED6* (*At3g21350*), SMP1 (*At1g65660*), *CHLM* (*At4g25080*), *GR1* (*At3g52115*), *protein kinase* (*At1g21590*), *MPK15* (*At1g73670*), *TUB8* (*At5g23860*), and *EF1α* (*At1g07920*). The microarray and tiling array data discussed in this article have been deposited in the National Center for Biotechnology Information's GEO (Edgar et al., 2002) and can be accessed through GEO Series accession numbers GSE039090, GSE41377, and GSE41378.

Supplemental Data

The following is available in the online version of this article.

Supplemental Figure 1. Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data; These Genes Showed Increased (A) and Reduced (B) Expression in *rcf1-1* without Cold Treatment as Determined by the ATH1 Microarray Analysis.

Supplemental Figure 2. Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data; These Genes Showed Increased Expression in *rcf1-1* after 12-h Cold Treatment as Determined by the ATH1 Microarray Analysis.

Supplemental Figure 3. Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data; These Genes Showed Reduced Expression in *rcf1-1* after 12-h Cold Treatment as Determined by the ATH1 Microarray Analysis.

Supplemental Figure 4. Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data; These Genes Showed Increased Expression in *rcf1-1* after 24-h Cold Treatment as Determined by the ATH1 Microarray Analysis.

Supplemental Figure 5. Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data; These Genes Showed Reduced Expression in *rcf1-1* after 24-h Cold Treatment as Determined by the ATH1 Microarray Analysis.

Supplemental Figure 6. Expression of Cold-Responsive Genes in Wild-Type and *rcf1-1* Seedlings, Subcellular Localization of RCF1, and *RCF1* Expression in *rcf1-2* and *rcf1-3* Plants.

Supplemental Figure 7. RCF1 Is an RNA-Dependent RNA Helicase That Is Not Involved in mRNA Export.

Supplemental Figure 8. STA1 and Brr2b Negatively Regulate*CBF2* Expression and Are Positive Regulators of Cold Tolerance.

Supplemental Figure 9. Mis-Spliced Transcripts of *EBF2* and *PUB45* in *rcf1-1* as Determined by RT-PCR and Hierarchical Clustering Analysis of Genes in Wild-Type Plants in Response to Cold Stress Treatments Based on Publically Available Gene Expression Data.

Supplemental Figure 10. Visualization of Intron Retention of *MED6* (*At3g21350*), *SMP1* (*At1g65660*), *CHLM* (*At4g25080*), *GR1* (*At3g52115*), *Protein Kinase* (*At1g21590*), and *MPK15* (*At1g73670*) under Unstressed Condition in *rcf1-1* with the Integrated Genome Browser.

Supplemental Figure 11. Expression of *CIR1*, *SPFH*, *SK12*, and *PRR5* in T-DNA knockouts of *CIR1*, *SPFH*, *SK12*, and *PRR5* (*cir1*, *spfh*, *prr5*, and *sk12*), *CIR1* Overexpression (*CIR1 OE*), *SPFH* Overexpression (*SPFH OE*), *SK12* Overexpression (*SK12 OE*), and *PRR5* Overexpression (*PRR5OE)* Seedlings Determined by qRT-PCR Analysis.

Supplemental Table 1. Genetic Analysis of *rcf1-1* (Wild Type [Female] × *rcf1-1* [Male] Cross).

Supplemental Table 2. Genes with Increased and Reduced Expression in *rcf1-1* without Cold Treatment as Determined by Microarray Analysis.

Supplemental Table 3. Common Genes with Altered Expression Levels in *rcf1-1* after 0-, 12-, and 24-h Cold Treatment and Common Genes with Altered Expression Levels in *rcf1-1* after 12- and 24-h Cold Treatment as Determined by Microarray Analysis.

Supplemental Data Set 1. Genes with Increased and Reduced Expression in *rcf1-1* after 12-h Cold Treatment as Determined by Microarray Analysis.

Supplemental Data Set 2. Genes with Increased and Reduced Expression in *rcf1-1* after 24-h Cold Treatment as Determined by Microarray Analysis.

Supplemental Data Set 3. Mis-Spliced Genes in *rcf1-1* as Determined by Tiling Array Analysis.

Supplemental Data Set 4. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

Q.G., J.W., and J.Z. designed the research. Q.G., J.W., Y.Z., C.C., C.J., and J.Z. performed the research. R.L. analyzed the tiling array data. Q.G. and J.Z. analyzed the remaining data. Q.G. and J.Z. wrote the article.

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