Lateral roots (LRs) are a major determinant of the root system architecture in plants, and developmental plasticity of LR formation is critical for the survival of plants in changing environmental conditions. In Arabidopsis thaliana, genetic pathways have been identified that regulate LR branching in response to numerous environmental cues, including some nutrients, salt, and gravity. However, it is not known how genetic components are involved in the LR adaptation response to cold. Here, we demonstrate that CYTOKININ RESPONSE FACTOR2 (CRF2) and CRF3, encoding APETALA2 transcription factors, play an important role in regulating Arabidopsis LR initiation under cold stress. Analysis of LR developmental kinetics demonstrated that both CRF2 and CRF3 regulate LR initiation. crf2 and crf3 single mutants exhibited decreased LR initiation under cold stress compared with the wild type, and the crf2 crf3 double mutants showed additively decreased LR densities compared with the single mutants. Conversely, CRF2 or CRF3 overexpression caused increased LR densities. CRF2 was induced by cold via a subset of the cytokinin two-component signaling (TCS) pathway, whereas CRF3 was upregulated by cold via TCS-independent pathways. Our results suggest that CRF2 and CRF3 respond to cold via TCS-dependent and TCS-independent pathways and control LR initiation and development, contributing to LR adaptation to cold stress.

INTRODUCTION

The plant root system is important for the anchorage of plants in soil and the uptake of water and nutrients (Hochholdinger and Zimmermann, 2008). The root system of dicotyledonous plants is made up of a primary root and lateral roots (LRs). LRs are a major determinant of the root system architecture in plants (Péret et al., 2009a). In Arabidopsis thaliana, LRs originate from founder cells formed from xylem pole pericycle cells primed in the basal meristem and undergo anticlinal and asymmetric division to generate a single layered primordium. These cells undergo further anticlinal and periclinal divisions to generate a dome-shaped LR primordium (LRP) that emerges from the primary root via cell separation (Parizot et al., 2008; Péret et al., 2009a, 2009b; Lavenus et al., 2013). The process of Arabidopsis LR development is critically regulated by auxin, mainly via two AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA)- AUXIN RESPONSE FACTOR (ARF) modules including SOLITARY-ROOT/IAA14-ARF7-ARF19 and BODENLOS/IAA12-ARF5 (Fukaki et al., 2002; Vanneste et al., 2005; De Smet et al., 2010).

Abiotic stresses including cold stress and the availability of nutrients are known to modulate the root system architecture of plants. Plants exposed to low temperature produce smaller root systems and roots of thinner diameter (Pahlavanian and Silk, 1988; Nagel et al., 2009). Low temperature reduces the biomass in the basal parts of root systems and in lateral roots and induces smaller branching angles between the primary and lateral roots (Nagel et al., 2009). Cold stress inhibits root basipetal auxin transport by reducing the trafficking of the auxin efflux carrier PIN2 and inhibiting the lateral relocalization of PIN3 in Arabidopsis (Shibasaki et al., 2009). Cold reduces both root meristem size and cell number, repressing the division potential of meristematic cells by reducing auxin accumulation (Zhu et al., 2015). Thus, cold stress inhibits root growth partly by modulating auxin synthesis, transport, and response.

Cytokinin and a subset of a two-component signaling (TCS) system are involved in cold stress signaling and response (Jeon et al., 2010; Shi et al., 2012; Jeon and Kim, 2013). In Arabidopsis, cytokinins use a multistep TCS system that comprises the three sensor histidine kinases ARABIDOPSIS HISTIDINE KINASEs (AHKs) AHK2, AHK3, and AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; Kakimoto, 2003), and three sensor histidine kinases ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS (AHPs) mediating the transfer of phosphoryl groups from AHKs to ARABIDOPSIS RESPONSE REGULATORs (ARRs) (To and Kieber, 2008), and three types of ARRs classified into type-A, type-B, and type-C (Pils and Heyl, 2009). The type-B ARRs (ARR1, 2, 10-14, and 18-21) are transcription factors that function as positive regulators of cytokinin signaling (Hwang et al., 2012). The type-A ARRs (ARR3-9 and 15-17) are rapidly and transiently induced by cytokinin treatment and function as negative regulators of cytokinin signaling (Kim et al., 2003; To et al., 2004; Lee et al., 2007; Hwang et al., 2012). The type-C ARRs (ARR22 and ARR24) have a domain structure similar to that of the type-A ARRs, but their expression is not induced by cytokinins (Kiba et al., 2004; Horák et al., 2008; Pils and Heyl, 2009). Although...
the role of type-C ARRs in cytokinin signaling is unclear, it has been proposed that ARR22 plays a positive role in the stress tolerance response (Kang et al., 2012). A subset of the Arabidopsis cytokinin TCS system is utilized for cold stress signaling and response (Jeon et al., 2010; Jeon and Kim, 2013; Zhu et al., 2015). ARR1 mediates the cold signal via AHP2, AHP3, or AHP5 from AHK2 and AHK3 to express a subset of type-A ARRs, regulating cold stress response and tolerance along with cytokinin (Jeon et al., 2010; Jeon and Kim, 2013). ARR1 and ARR12 are also involved in the inhibition of Arabidopsis root growth by low temperature (Zhu et al., 2015).

Analysis of the cold transcriptome affected by ahk2 ahk3 mutations revealed a cold-responsive gene network regulated downstream of AHK2 and AHK3. CYTOKININ RESPONSE FACTOR2 (CRF2), encoding a member of the APETELA2 (AP2) ETHYLENE RESPONSE FACTOR (ERF) transcription factors, was found to be downregulated by ahk2 ahk3 mutations in response to cold, indicating that cold-responsive expression of CRF2 is regulated downstream of AHK2 and AHK3 (Jeon and Kim, 2013). Some CRF members, such as CRF1, CRF2, and CRF5, play a role in the development of the cotyledon, leaf, and embryo (Rashotte et al., 2006). CRF2, CRF5, and CRF6, but not CRF1, CRF3, or CRF4, were upregulated by cytokinins (Rashotte et al., 2006). Microarray analysis of arr1, 12, crf1,2,5, and crf,3,6 with or without cytokinin benzyladene-nine revealed that some CRF members act in parallel with type-B ARRs to mediate cytokinin-regulated gene expression and also coregulate the target genes with type-B ARRs (Rashotte et al., 2006). The AP2/ERF family transcription factors have been implicated in the response to plant hormones and as mediators of stress responses and developmental processes (Mizoi et al., 2012; Licauisi et al., 2013).

Here, we show that cold induces the expression of CRF2 and CRF3, which are involved in LR initiation and LR formation, via TCS-dependent and TCS-independent signal transduction pathways, respectively, and that CRF2 and CRF3 promote LR initiation under cold stress. Cold-responsive CRF2 expression is affected by mutations in AHK2 and AHK3 as well as in AHP2, AHP3, and AHP5, indicating that the TCS pathway is involved in regulating CRF2 expression in response to cold. We further showed that ARR1 directly regulates CRF2 expression by binding to the CRF2 promoter. By contrast, the cold response of CRF3 expression is not altered by mutations in the TCS signaling components. Mutations in CRF2 and CRF3 cause a delay in LR initiation and a reduction in LR formation, whereas overexpression of CRF2 or CRF3 in Arabidopsis enhances LR initiation and LR formation. The crf2 and crf3 single and double mutants display a hypersensitive response in LR initiation events to cold stress compared with that of the wild type, whereas overexpression of CRF2 or CRF3 enhances LR initiation under cold stress. Based on these results, we propose that CRF2 and CRF3 integrate the environmental cold signal into LR development and mediate the adaptation to cold stress to influence changes in root system architecture and limit the negative effects of cold on root growth.

RESULTS

CRF2 and CRF3 Respond to Cold

The Arabidopsis CRF gene family comprises 12 members (Rashotte and Goertzen, 2010; Zwack et al., 2012). We first examined the expression profiling of CRFs using the Genevestigator Web tool, which provides a summary of gene expression responses to a variety of stimuli, in order to identify the CRF genes that are responsive to cold. Figure 1A shows the responses of CRFs to phytohormones and abiotic stresses including cold. CRF2, CRF3, CRF6, and CRF10 exhibited cold response, and in particular, CRF2 and CRF3, a close homolog to CRF2, showed strong induction by cold (Figure 1A). We determined cold-responsive expression of CRF2 and CRF3 by conducting RT-qPCR for the plants treated with cold for varying times. CRF2 and CRF3 showed a transient change in gene expression with maximum response occurring with 4 h of cold treatment (Figure 1B). CBF1 was used as a cold marker gene to confirm the effect of the cold treatment with regard to the induction of gene expression. Both CRF2 and CRF3 exhibited significant cold-responsive gene expression in both the shoot and the root, although CRF2 showed higher levels of cold response in the shoot compared with that in the root (Figure 1C).

CRF2 and CRF3 Are Expressed via Different Cold Signaling Pathways

To determine whether TCS components are involved in cold-inducible expression of CRF2 and CRF3, we analyzed the expression of CRF2 and CRF3 in ahk, ahp, and type-B arr mutants using RT-qPCR. Plants were treated with cold for 4 h, corresponding to peak expression in wild-type plants (Figure 1B). The ahk2 ahk3 double mutants exhibited significantly reduced CRF2 expression in response to cold compared with that in the wild type (Figure 2A). The ahp2 ahp3 ahp5 triple mutants also showed significantly reduced cold-responsive expression of CRF2 and the ahp1 ahp2 ahp3 ahp4 ahp5 quintuple mutants displayed more reduced CRF2 expression than that in the triple mutants (Figure 2B). All of the mutants are in the Col-0 accession except for ahp1-1, which is in the Wassilewskija-2 background. Although there are multiple genetic differences between Col-0 andWs that could influence the interaction with the quintuple mutant and CRF2 expression, this is unlikely given the comparison to each respective wild type (Figure 2B). CRF2 expression was also reduced in the type-B arr mutants such as arr1, arr10, and arr12 single mutants and arr1 arr10, arr1 arr12, and arr10 arr12 double mutants and even more reduced in arr1 arr10 arr12 triple mutants, indicating that ARR1, ARR10, and ARR12 in part redundantly regulate CRF2 expression in response to cold (Figure 2C). By contrast, CRF3 expression in response to cold was not altered in any of these TCS mutants compared with that of the wild type. These results indicated that TCS is involved in mediating the cold signal for expressing CRF2, whereas cold-responsive expression of CRF3 occurs independently of the TCS system.

CRF protein subcellular localization assays have previously shown that cytokinin rapidly induces the relocalization of CRF2, CRF3, and CRF6 into the nucleus and that both AHPs and cytokinin receptors are required for the cytokinin-regulated movement of CRF2 into the nucleus (Rashotte et al., 2006). We analyzed the subcellular distribution of EGFP:CRF2 and EGFP:CRF3 in the wild-type Arabidopsis mesophyll protoplasts without or with cold treatment by confocal scanning laser microscopy, showing that both CRF2 and CRF3 were localized in both the cytosol and the
nucleus and that cold treatment did not affect the subcellular distribution of these two CRFs (Supplemental Figure 1).

ARR1 Regulates CRF2 Expression by Directly Binding to the CRF2 Promoter

As ARR1 in combination with ARR10 and ARR12 are redundantly involved in cold-responsive expression of CRF2 (Figure 2C) and ARR1 has been shown to play a key role in cold-responsive expression of type-A ARRs (Jeon and Kim, 2013), we investigated whether ARR1 directly regulates CRF2 expression. We generated transgenic Arabidopsis expressing ARR1 tagged with 10 copies of MYC epitopes in frame with ARR1 N terminus in arr1-3 mutants (Pro35S:10xMYC:ARR1/arr1-3) to test whether ARR1 expression rescues the reduced cold-responsive expression of CRF2 in arr1 mutants compared with that in the wild type (Supplemental Figure 2). The arr1 mutants and Pro35S:10xMYC:ARR1/arr1-3 plants were treated with cold for 0, 2, 4, or 24 h, and CRF2 expression was determined by RT-qPCR compared with that in the wild type (Figure 3A). arr1 mutants showed reduced expression of CRF2 after 2 and 4 h of cold treatment, whereas Pro35S:10xMYC:ARR1/arr1-3 plants exhibited wild-type response of CRF2 expression to cold. However, CBF1 was expressed at the same levels in response to cold in wild-type, arr1, and Pro35S:10xMYC:ARR1/arr1-3 plants. These results demonstrated that ARR1 positively regulates CRF2 expression in response to cold.

To prove whether ARR1 upregulates CRF2 expression via the CRF2 promoter, we used Arabidopsis protoplast transient gene expression assays with a reporter plasmid harboring the CRF2 promoter fused to LUC (ProCRF2:LUC) and an effector plasmid harboring ARR1 under the control of the CaMV 35S promoter (Pro35S:V4xHA:ARR1). ARR1 expression resulted in a 70-fold enhancement of LUC expression from ProCRF2:LUC, whereas the coexpression of ProCRF2:LUC with a negative control, Pro35S:3xHA:EGFP, did not enhance LUC expression (Figure 3B). This result demonstrated that ARR1 upregulates CRF2 expression through the CRF2 promoter. To examine whether ARR1 directly upregulates CRF2 expression, we analyzed CRF2 expression in transgenic Arabidopsis overexpressing ARR1DDK fused with the glucocorticoid receptor hormone binding
domain (GR), but without the ARR1 receiver domain (Pro35S:ARR1 ΔDDK:GR) in a dexamethasone (DEX)-inducible manner (Jeon and Kim, 2013). DEX treatment of Pro35S:ARR1 ΔDDK:GR plants induced the expression of CRF2 by 3-fold compared with that in mock-treated transgenic plants (Figure 3C). While the treatment of cycloheximide, a protein synthesis inhibitor, of Pro35S:ARR1 ΔDDK:GR plants resulted in increased CRF2 expression, cycloheximide did not prevent the DEX-induced expression of CRF2. These results indicated that CRF2 is a primary response gene directly regulated by ARR1 without new protein synthesis.

We identified eleven putative ARR1 binding sites harboring the conserved 5’-AGATT-3’ sequence element in the 2-kb CRF2 promoter region relative to the AUG codon (Figure 3D). To determine whether ARR1 can directly bind to these ARR1 binding sites in the CRF2 promoter, we first performed an electrophoretic mobility shift assay (EMSA) using truncated ARR1 recombinant protein encompassing 300 amino acids from the N terminus fused with GST. We selected a particular region harboring the ARR1 binding site (indicated by the arrowhead A in Figure 3D) and prepared 29-mer of oligonucleotide DNA probe (probe A) from the CRF2 promoter sequences for EMSA. GST-ARR1 was shown to.

Figure 2. Expression of CRF2 and CRF3 in Two-Component Signaling Mutants in Response to Cold.

Expression of CRF2 and CRF3 in wild-type (Col-0 or Ws as indicated) and ahk2-2 ahk3-3 double mutants (A), ahp triple and pentuple mutants (B), and arr single, double, and triple mutants (C) treated for 0 or 4 h at 1°C. Ten-day-old plants were treated for 4 h at 1°C and subjected to RT-qPCR analysis. Relative fold changes were plotted after normalization to ACTIN7. Bars with different letters indicate significance at P < 0.01 by two-way ANOVA and the Tukey’s honestly significant difference test (Supplemental Figure 9). All panels show mean ± se values determined from n = three biological replicates (each biological replicate was estimated as the average of two technical RT-qPCR replicates).
Figure 3. ARR1 Regulates CRF2 Expression by Directly Binding to the CRF2 Promoter.

(A) Expression analysis of CRF2 in arr1-3 mutants and Pro35S::10xMYC::ARR1/arr1-3 plants in response to cold. Mean ± se values determined from n = three biological replicates (each biological replicate was estimated as the average of two technical RT-qPCR replicates). Relative fold change represents the ratio relative to the transcript level of the wild type at 0 h. Bars with different letters indicate significance at P < 0.01 by two-way ANOVA and the Tukey's honestly significant difference test (Supplemental Figure 9).

(B) Transcriptional activation by ARR1 via the CRF2 promoter in Arabidopsis protoplasts. The values on the y axis represent the relative light units of LUC activity after normalizing to GUS activity. Mean ± se values determined from n = three biological replicates (each biological replicate was estimated as the average of two technical RT-qPCR replicates). Asterisks indicate significance at P < 0.001 using Student's t test when compared to controls.

(C) Activation of CRF2 expression by ARR1:GR in the presence of cycloheximide. Ten-day-old Pro35S::ARR1::DDK:GR plants were incubated with DEX, cycloheximide, or DEX and cycloheximide for 2 h under the light. Mean ± se values determined from n = three biological replicates (each biological replicate was estimated as the average of two technical RT-qPCR replicates). Bars with different letters indicate significant differences at P < 0.01 by one-way ANOVA with the Tukey's honestly significant difference test (Supplemental Figure 9).

(D) Schematic diagram of CRF2. The nucleotide sequences, 5′-AGATT-3′, to which ARR1 binds, are indicated by circles in the promoter region. The arrowhead indicates the probe region used for EMSA. P1, P2, P3, and P4 indicate the promoter regions for the ChIP assays (F). The black box indicates exon. P1, -1171 to -1001 nucleotides from AUG; P2, -738 to -552; P3, -270 to -128; P4, -44 to +79.

(E) EMSA of ARR1 with a CRF2 promoter DNA probe. EMSA was performed with 250 ng of GST:ARR1 and 400 fmol of the indicated DNA probe. In the left panel, EMSA was performed with increasing quantities of unlabeled probe A at 10-, 50-, 100-, or 200-fold relative to the quantity of radiolabeled probe A. In the right panel, EMSA was performed with increasing quantities of unlabeled probe B at 10-, 50-, 100-, or 200-fold relative to the quantity of radiolabeled probe B.
bind to probe A (Figure 3E). This binding was easily outcompeted with increasing quantities of specific probe A but was not affected by a nonspecific probe B. We also conducted chromatin immunoprecipitation (ChIP) assays using two lines of ARR1OX/arr1-3 plants in four different regions from P1 to P4 in the CRF2 promoter (Figure 3D) with or without cold treatment for 3 h (Figure 3F). The ChIP assay results showed that ARR1 binds to the CRF2 chromatin primarily in the P1 and P2 regions harboring the conserved ARR1 binding sites, whereas ARR1 did not bind to the P3 and P4 regions which do not have an ARR1 binding site. However, cold treatment did not affect the binding of ARR1 to the CRF2 chromatin. Taken together, these results demonstrated that ARR1 directly binds to the CRF2 promoter in vitro and in vivo and that cold does not alter the binding of ARR1 to the CRF2 chromatin.

**CRF2 and CRF3 Promote Lateral Root Formation**

To determine tissue-specific expression patterns of CRF2 and CRF3, we generated transgenic Arabidopsis harboring the 2-kb promoter region of CRF2 or CRF3 fused to a EGFP:GUS reporter gene and conducted histochemical GUS assays for these transgenic plants. ProCRF2:EGFP:GUS (CRF2:GUS) plants showed strong GUS expression in both the shoot and root regions, the vascular tissue of leaves and the root, mature LRs, and LRP (Figures 4A to 4E). ProCRF3:EGFP:GUS (CRF3:GUS) plants showed GUS expression patterns similar to those of ProCRF2: EGFP:GUS plants except for GUS expression in the reproductive organs such as the anther and stigma (Figures 4F to 4L). As we detected strong GUS expression in LRP and LR in both transgenic GUS reporter plants, GUS expression of these transgenic plants during LRP development was determined at given developmental stages based on the classification by Malamy and Benfey (1997). In the case of ProCRF2:EGFP:GUS plants, GUS expression was detectable at stage I LRP and increased during LRP development up to LR emergence (Figures 4M). By contrast, GUS expression in ProCRF3:EGFP:GUS plants was nearly undetectable at stage I LRP but gradually increased from stage III LRP up to LR emergence (Figure 4N). Relatively stronger GUS expression was detected up to stage V in ProCRF2:EGFP:GUS plants than in ProCRF3:EGFP:GUS plants. These GUS expression patterns indicated the potential role of CRF2 and CRF3 during LR development.

In order to investigate the biological role of CRF2 and CRF3, we isolated crf2-2, crf3-2, and crf3-3 Arabidopsis T-DNA insertion mutants and generated crf2-2 crf3-2 and crf2-2 crf3-3 double mutants. As shown in Supplemental Figure 3, the crf2-2, crf3-2, and crf3-3 mutants have T-DNA insertions in exon region of CRF2 or CRF3 and displayed undetectable expression of CRF2 or CRF3 by both RT-PCR and RT-qPCR analysis, demonstrating that these crf mutants are null. We also produced transgenic Arabidopsis overexpressing CRF2 or CRF3 tagged with three copies of HA epitopes (Pro35S:3xHA:CRF2 or Pro35S:3xHA:CRF3) (Supplemental Figure 3). Analysis of the morphological changes in crf2 and crf3 single and double mutants and overexpression lines compared with wild-type revealed root phenotypes (Figure 5). Primary root lengths of crf2 and crf3 single mutants were significantly shorter than those of the wild type but were similar to those of crf2 crf3 double mutants (Figures 5A and 5B), indicating that CRF2 and CRF3 are involved in primary root development through the same genetic pathway. We found that while the LR density of crf2-2 single mutants (2.06 ± 0.40) was similar to that of the wild type (2.08 ± 0.34), those of crf3-2 (1.61 ± 0.28) and crf3-3 (1.67 ± 0.34) single mutants were reduced compared with that of the wild type, and those of crf2-2 crf3-2 (1.11 ± 0.39) and crf2-2 crf3-3 (1.27 ± 0.40) double mutants were further reduced compared with those of crf3 single mutants (Figure 5C). The reduction in LR density of crf3-2 mutants was significantly rescued by expressing CRF3 under the control of the CRF3 promoter (Supplemental Figure 4C). These results indicated that CRF3 plays a role in LR formation and that CRF2 acts with CRF3 to regulate LR formation. Three different transgenic lines overexpressing CRF2 or CRF3 displayed a significant increase in LR density compared with that of the wild type, whereas primary root lengths were slightly reduced in all transgenic overexpression lines (Figures 5D to 5F). These results together demonstrated that both CRF2 and CRF3 positively regulate LR formation.

**CRF2 and CRF3 Regulate LR Initiation**

To identify the steps of LR development in which CRF2 and CRF3 act, we enumerated LR densities from stage I to stage VII. Although the mean LR density at stage I of crf2-2 mutants (0.71 ± 0.29) was insignificantly lower than that of the wild type (0.83 ± 0.16), the mean LR densities at stage I of crf3-2 (0.4 ± 0.2) and crf2-2 crf3-3 (0.47 ± 0.17) mutants were significantly lower than that of the wild type (Figure 6A). We also showed that the reduction in LR density at stage I of crf3-2 mutants was significantly rescued by expressing CRF3 under the control of the CRF3 promoter (Supplemental Figure 5). Two lines of transgenic Arabidopsis overexpressing CRF2 or CRF3 displayed increased LR densities at stages I and II. These results indicated that both CRF2 and CRF3 are involved in promoting LR initiation. As crf2-2 mutants displayed a similar number of primordia at stage I compared with that of the wild type, and since the double mutations in crf2 and crf3 did not further increase a delay in LR initiation caused by crf3 single mutation, we conducted a LR induction experiment which allows the direct analysis of LR development kinetics rather
than the analysis of the LRP and LRs at a given developmental stage of plants (Péret et al., 2012; Lee and Kim, 2013). We applied a gravitropic stimulus inducing initiation of LRs to the wild type, crf mutants, and transgenic overexpression lines grown vertically for 3 d by rotating the agar plate through 90°. We then measured the number of newly developed primordia on the convex side of the curves at 30 and 54 h postgravitropic induction (pgi) and determined the relative distribution of the LRP at stages I to VIII. Both crf2-2 and crf3-2 mutants showed delayed LRP development in response to the gravitropic stimuli, and crf3-2 crf2-2 double mutants displayed more delayed LRP development compared with that of the corresponding single mutants (Figure 6B). Moreover, the crf3-2 and crf2-2 crf3-2 mutants displayed 20 and 35% blockage (percentage of plants with no primordium) respectively, of LR initiation events compared with that of the wild type at 30 h pgi (Figure 6B). Distribution of the LRP at stages I to VIII in the crf2-2, crf3-2, and crf2-2 crf3-2 mutants showed that developmental transitions from stages I to III were inhibited to some extent at 30 h pgi and the LRP development of these crf mutants was delayed at 54 h pgi compared with that of the wild type. By contrast, overexpression of CRF2 or CRF3 in transgenic Arabidopsis stimulated LRP developmental processes from initiation to emergence of LRP at both 30 and 54 h pgi compared with that of the wild type. Taken together, these results demonstrated that CRF2 and CRF3 positively control LR initiation in different genetic pathways and that crf3 exhibits a stronger genetic effect on LR development than does crf2.

**CRF2 and CRF3 Are Required for LR Initiation under Cold Stress**

As CRF2 and CRF3 respond to cold (Figure 1) and play a role during LR initiation and LR development (Figures 5 and 6), we sought to characterize the role of CRF2 and CRF3 during LR development when plants are exposed to cold temperatures. We first determined cold response of CRF2 and CRF3 during LRP development and in LRs using GUS reporter transgenic plants. Cold treatment for 8 h enhanced GUS expression in LRP from stage III to the emerged LRs and elongated LRs of both ProCRF2:EGFP::GUS and ProCRF3:EGFP::GUS plants (Figures 7A and 7B). Cold also enhanced GUS staining in leaves of both ProCRF2:EGFP::GUS and ProCRF3:EGFP::GUS seedlings (Supplemental Figure 6), which is consistent with the RT-qPCR data (Figure 1C).

As CRF2 and CRF3 respond to cold during LR development and play a role in LR initiation, we investigated whether CRF2 and
DISCUSSION

As LRs are the major determinant of the root system architecture in plants, the developmental plasticity of LR formation is important for the survival of plants to continually changing environmental conditions. In Arabidopsis, the developmental and molecular mechanisms of LR formation have been well characterized (Lavenus et al., 2013) and the genetic pathways regulating LR branching in response to environmental cues such as nutrients including nitrate and phosphate, salt, and gravity have been studied. The results showed that LR initiation events in the wild type decreased with increasing time of cold treatment at 4°C and that crf2-2 mutants exhibited a further decrease in LR initiation events compared with those of the wild type. The effects of cold stress on the LR initiation events of crf3-2 mutants were more severe than those of crf2-2 mutants. The crf2-2 crf3-2 double mutants exposed to cold stress displayed additively reduced LR initiation events compared with those of the corresponding crf single mutants. Overexpression of CRF2 or CRF3 in transgenic Arabidopsis prevented the decrease in LR initiation events caused by cold stress to some extent compared with the wild type (right panel of Figure 7C). We further showed that reduction in LR initiation events of crf2-2 or crf3-2 mutants under cold stress was significantly rescued by expressing CRF2 or CRF3 under the control of their own promoter in the corresponding mutants (Figure 7D). These results suggested that CRF2 and CRF3 are necessary for LR initiation via different genetic pathways when plants are exposed to cold stress.

CRF3 are required for LR initiation when plants are exposed to cold stress. To this end, 3-d-old wild-type, crf2-2, crf3-2, crf2-2 crf3-2, CRF2OX, and CRF3OX transgenic Arabidopsis were subjected to a 90° gravitropic stimulus and treated for 8, 10, or 12 h at 4°C and then placed back to 23°C while being subjected to a 90° gravitropic stimulus, and LR initiation events were then measured at 30 h pgi (Figure 7C). To detect significant differences between genotypes and treatments with their respective controls, we first used two-way ANOVA, combining different cold treatment incubation times with different genotypes, but this did not capture significant differences, due to the amount of intrinsic variation in LR development between genotypes. As an alternative statistical analysis, we conducted one-way ANOVA to capture significant differences among different genotypes for the given cold treatment time as indicated by bars with different capital letters or with primed different capital letters and then conducted another one-way ANOVA to capture significant differences among different genotypes for the given cold treatment time as indicated by bars with different small letters or with primed different small letters. The SD values were determined from 30 seedlings. Asterisks indicate significant differences at P < 0.001 using Student’s t test when comparing to the wild type.

(A) Representative seedlings of the wild-type, crf2-2, crf3-2, crf3-3, crf2-2 crf3-2, crf2-2 crf3-3, and Pro35S:3xHA:CRF2 and Pro35S:3xHA:CRF3 Transgenic Arabidopsis.

(B) Root lengths of wild-type, crf2-2, crf3-2, crf3-3, crf2-2 crf3-2, and crf2-2 crf3-3 mutants. Bars = 1 cm. Mean ± so values were determined from three biological replicates of 28 seedlings. Bars with different letters indicate significant differences at P < 0.05 by one-way ANOVA with the Tukey’s honestly significant difference test (Supplemental Figure 9).

(C) LR densities of wild-type, crf2-2, crf3-2, crf3-3, crf2-2 crf3-2, and crf2-2 crf3-3 mutants. LR numbers (#) per unit primary root length (cm) measured from plants grown vertically for 8 d were plotted. Mean ± so values were determined from three biological replicates of 28 seedlings. Bars with different letters indicate significant differences at P < 0.01 by one-way ANOVA with the Tukey’s honestly significant difference test (Supplemental Figure 9).

(D) Representative seedlings of wild-type, Pro35S:3xHA:CRF2 (CRF2OX), and Pro35S:3xHA:CRF3 (CRF3OX) transgenic plants. Plants were grown vertically for 8 d. Bars = 1 cm.

(E) Root lengths of wild-type, Pro35S:3xHA:CRF2, and Pro35S:3xHA:CRF3 transgenic plants. Plants were analyzed as described in the Figure 5B legend. Mean ± so values were determined from 30 seedlings. Asterisks indicate significant differences at P < 0.001 using Student’s t test when comparing to the wild type.

(F) LR densities of wild-type, Pro35S:3xHA:CRF2, and Pro35S:3xHA:CRF3 transgenic plants. Plants were analyzed as described in the Figure 5C legend. Mean ± so values were determined from 30 seedlings. Asterisks indicate significant differences at P < 0.001 using Student’s t test compared to the wild type.

Figure 5. Root Lengths and LR Densities of the Wild Type, crf2-2, crf3-2, crf3-3, crf2-2 crf3-2, crf2-2 crf3-3 Mutants, and Pro35S:3xHA:CRF2 and Pro35S:3xHA:CRF3 Transgenic Arabidopsis.
identified (Dastidar et al., 2012; Tian et al., 2014). While cold is one of the critical environmental conditions that limits plant growth and development as well as crop yield (Jeon and Kim, 2013), it is not known how genetic components are involved in LR adaptation response to cold. This study demonstrated that CRF2 and CRF3 play an important role during LR development in Arabidopsis and also integrate the cold signal into LR development for an adaptation response to cold stress via signaling pathways as depicted in the model (Figure 8). A recent study showed that CRF2 and CRF6 transcriptionally control genes encoding PIN-FORMED (PIN) auxin transporters such as PIN1 and PIN7, providing a crosstalk component that fine-tunes auxin transport capacity downstream of cytokinin signaling to control plant growth and development (Simásková et al., 2015). Therefore, CRF2 and CRF3 gate endogenous hormone signals, such as cytokinin and auxin, as well as the environmental cold signal, providing adaptation versatility to the plants under cold stress. It remains to be determined whether CRF2 promotes LR initiation under cold stress through upregulation of PIN gene expression.

This work showed that a subset of the cytokinin TCS system is utilized for cold signaling to express CRF2. A significant change in cold-responsive expression of CRF2 in ahk2 ahk3 double mutants was detected (Figure 2A), indicating that AHK4 may be involved in the cold response of CRF2 in addition to AHK2 and AHK3.
However, ahp1,2,3,4,5 pentuple mutants also exhibited significant levels of CRF2 expression in response to cold (Figure 2B), suggesting that an additional gene regulatory pathway other than the TCS pathway is involved in cold response of CRF2. None of the TCS mutants used in this study affected cold-responsive expression of CRF3 (Figure 2), showing that the cold response of CRF3 occurs independently of the TCS system. A microarray analysis conducted on the Arabidopsis mutant harboring
a dominant mutation in INDUCER OF C-REPEAT/DEHYDRATION RESPONSE ELEMENT BINDING FACTOR EXPRESSION1 (ice1) indicated that CRF3 may be regulated by ICE1 (Lee et al., 2005). However, our analysis with loss-of-function mutants ice1 and ice2 (Fursova et al., 2009) showed that these mutations only slightly affected cold-responsive expression of CRF3 (Supplemental Figure 7), suggesting that a gene regulatory network other than ICE1 may be involved in cold response of CRF3.

arr1,10,12 triple mutations reduced cold-responsive expression of CRF2 by 60% compared with that in the wild type (Figure 2C), indicating that ARR1, ARR10, and ARR12 are the main transcription factors regulating the cold response of CRF2. To identify if these type-B ARRs directly regulate CRF2 expression in response to cold, we used a variety of molecular approaches such as transient gene expression assays, DEX-inducible nuclear translocation of GR-fused CRF2, EMSA, and ChIP assays (Figure 3), demonstrating that ARR1 directly regulates CRF2 expression by binding to the CRF2 promoter. The ChIP assay results demonstrated that cold did not enhance the ARR1 binding to the CRF2 promoter in the chromatin (Figure 3F), indicating that cold does not alter the DNA binding activity of ARR1 in vivo but rather activates the transactivation potential of ARR1. It will be interesting to determine whether the phoshorelay from AHKs via AHPs in response to cold controls the transcriptional activities of ARR1 and other type-B ARRs. SELEX and EMSA experiments have shown that ARR1, ARR2, and ARR11 bind to the same or a very similar nucleotide sequence motif, 5'-[(A/G)GAT(T/C)]-3' (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). Experiments using protein binding microarrays have shown that ARR11 and ARR14 preferentially bind to 5'-AGATACG-3' or 5'-AGATCTT-3' or similar sequences (Franco-Zorrilla et al., 2014). Thus, ARR10 and ARR12 or other type-B ARRs may directly promote CRF2 expression through binding to the core cytokinin response motif sequence that resides in the CRF2 promoter region.

Analysis of a variety of root transcript profiling data sets showed that a set of 1920 genes display transcriptional changes in the xylem pole pericycle cells during lateral root initiation (Swarup et al., 2008). This set of genes included CRF2 but not CRF3 (Swarup et al., 2008). Our GUS expression analysis of ProCRF2::EGFP::GUS plants showed variable staining of GUS at stage I LRP, probably due to low and/or dynamic expression, as shown in Figures 4M and 7A. Variable expression of GUS at stage III LRP in ProCRF3::EGFP::GUS plants was also noted (Figures 4M and 7B). These results may be due to the stochastic nature of CRF2 and CRF3 expression. Relatively large variations in LRP densities were noticed in this study (Figure 6A) as well as in the report by Swarup et al. (2008). Stochastic fluctuations in gene expression have been proposed to underlie the phenotypic variation and cellular variation in eukaryotic organisms even in fixed genetic and environmental contexts (Blake et al., 2003; Raj et al., 2010). Such stochastic effects in gene expression could have implications in LR development, as they might generate variation in the distribution of LR at a variety of different developmental stages.

Analysis of the expression profiling of CRFs in response to various plant hormones and in a variety of mutant backgrounds using the Genevestigator Web tool showed that CRF2 weakly responds to auxin but strongly responds to cytokinins and is unresponsive to cytokinins in ahk2 ahk3 mutants (Figure 1A; Supplemental Figure 8), indicating that cytokinin may regulate CRF2 expression through the AHK2- and AHK3-mediated TCS pathway. However, cytokinins are known to negatively control LR formation by preventing the establishment of an auxin gradient required for LR formation and to act as a paracrine signal regulating LR spacing (Riefler et al., 2006; Laplaze et al., 2007; Bielach et al., 2012; Chang et al., 2015). Although the role of CRF2 during the inhibitory action of cytokinin for LR formation remains to be determined, it is plausible to hypothesize that cold activates a subset of the TCS pathway to express CRF2. CRF3 responds to auxin and is unresponsive to auxin in arf7, arf19, and arf7 arf19 mutant backgrounds (Figure 1A; Supplemental Figure 8),
suggesting that auxin may regulate CRF3 expression through ARF7 and ARF19. The previous studies showed that cold temperature inhibits root basipetal auxin transport and reduces auxin accumulation, limiting root growth in Arabidopsis (Shibasaki et al., 2009; Zhu et al., 2015), indicating that cold temperature inhibits auxin transport, biosynthesis, and response in the root. We speculate that the expression of CRF2 and CRF3 in response to cold may be an adaptation mechanism of plants under cold stress, enabling LR initiation and development to overcome the auxin-mediated cold-induced inhibition of root growth, thus influencing changes in root system architecture in response to cold.

Several transcription factors, including LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE (LBD/ASL) proteins such as LBD16, LBD18, LBD29, and LBD33, are regulated at the transcriptional level downstream of Aux/IAA-ARF modules in response to auxin to control LR development. While these factors are regulated at the transcriptional level downstream of Aux/IAA-ARF modules in response to auxin to control LR development, we have noted that changes in root system architecture in response to cold.

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**METHODS**

**Plant Materials and Growth Conditions**

Wild-type and mutant lines of Arabidopsis thaliana were in the Columbia (Col-0) ecotype except for ahp1-1 in the Wassilewskija (Ws) ecotype. Plants were grown on 0.5× MS agar plates or in soil at 23°C with a 16-h photoperiod. The light intensity was ~120 μmol m⁻² s⁻¹. The crf2-2 (SAIL371_D04), crf2-3 (SAIL325_H03), and crf3-3 (SALK_138253) mutants were obtained from the ABRC. The homozygous T-DNA insertion mutant lines were identified by PCR using the primers shown in Supplemental Table 1. The crf2-2 crf2-3 and crf2-2 crf3-3 double mutants were generated by crossing crf2-2 (male) with crf3-2 or crf3-3 (female), and the resultant isolated homozygous lines were verified by PCR. The null mutations of crf2-2, crf2-3, crf3-2, crf3-3, and crf2-2 crf3-3 were further verified by RT-PCR and RT-qPCR analysis. The ahk2-2 ahk3-3, ahp2-1 ahp3 ahp3-2, ahp1-3, ahp10-5, ahp12-2, ahp1-3 ahp1-3 ahp12-5, ahp1-3 ahp12-2, ahp1-3 ahp10-5 ahp12-2, and ahp1-3 ahp10-5 ahp12-2 mutants have been described previously (Jeon and Kim, 2013). ahp1-1 2-1, 2-1, 4 was crossed to ahp2-1, 2-1, 3, 5-2 to generate ahp1-1, 2-1, 2-1, 4, 5-2 quintuple mutants. To generate transgenic plants over-expressing the CRF2 and CRF3 genes, the full-length CRF2 and CRF3 coding regions were isolated by PCR from Arabidopsis cDNA and inserted into pDONR221 (Invitrogen) using the Gateway BP Clonase II enzyme mix (Invitrogen). These constructs were inserted into pGWB515 vector (Nakagawa, Shimane University, Japan) using the Gateway LR Clonase II enzyme mix (Invitrogen). The Pro35S:3xHA:CRF2 and Pro35S:3xHA:CRF3 constructs, respectively. The Pro35S:3xHA:CRF2 and Pro35S:3xHA:CRF3 constructs were introduced into Arabidopsis using the vacuum infiltration Agrobacterium tumefaciens-mediated transformation method, and T3 homozygous transgenic plants were obtained. To generate the promoter-GUS transgenic Arabidopsis, the promoter region of CRF2 encompassing ~2005 to ~1 bp relative to the AUG initiation codon and the promoter region of CRF3 encompassing ~2021 to ~1 bp relative to the AUG initiation codon were isolated by PCR from Arabidopsis genomic DNA and inserted into pDONR221 (Invitrogen) using the Gateway BP Clonase II enzyme mix (Invitrogen) to yield pDONR221:ProCRF2 and pDONR221:ProCRF3, respectively. These constructs were inserted into the pBGWFS7 vector using the Gateway LR Clonase II enzyme mix (Invitrogen). The ProCRF2:GFP:GUS and ProCRF3:GFP:GUS constructs were transformed into Arabidopsis, and T3 homozygous transgenic plants were obtained. To generate ProCRF2: CRF2:3xHA:crf2-2 or ProCRF3:CRF3:3xHA:crf3-3 Arabidopsis, we isolated the CRF2 genomic DNA fragment encompassing from ~2005 to ~1032 bp relative to AUG initiation codon that includes the promoter and the full-length CRF2 coding region and the CRF3 genomic DNA fragment encompassing from ~2021 to ~1065 bp region that includes the promoter and the full-length CRF3 coding region from the Arabidopsis genomic DNA by PCR using Pfu polymerase (Stratagene). These PCR products were then inserted into pDONR221 (Invitrogen) by BP recombination reaction using Gateway BP Clonase II Enzyme mix (Invitrogen) and subcloned into the pGWB513 vector (Nakagawa, Shimane University, Japan) by LR recombination reaction, yielding the ProCRF2:CRF2:3xHA:crf2-2 or ProCRF3:CRF3:3xHA:crf3-3 Arabidopsis constructs. These constructs were then transformed into crf2-2 or crf3-2 mutants by Agrobacterium-mediated transformation, and T3 homozygous transgenic mutant plants were obtained. To generate Pro35S:10xMYC:ARR1:acrfl-3 transgenic Arabidopsis plants, the full-length ARR1 coding regions were amplified by PCR using Pfu polymerase (Stratagene) and inserted into pDONR221 (Invitrogen) by BP recombination reaction using Gateway BP Clonase II Enzyme mix (Invitrogen). This construct was subcloned into the pGWB521 vector (Nakagawa, Shimane University, Japan) by LR recombination reaction, yielding Pro35S:10xMYC:ARR1:acrfl transgenic Arabidopsis plants. These transgenic plants were produced using T3 homozygous transgenic mutant plants. All constructs were confirmed via DNA sequencing prior to plant transformation. The 5’S:ARR1:DDK:GR construct was generously provided by Takashi Aoyama (Sakai et al., 2000) and confirmed via genotyping prior to use. Oligonucleotides and PCR conditions are provided in Supplemental Table 1.

**RNA Isolation, RT-PCR, and RT-qPCR**

The Arabidopsis plants were immediately frozen in liquid nitrogen following treatment and stored at ~80°C. Total RNA was isolated from frozen Arabidopsis samples using TRI reagent (Molecular Research Center). RT-PCR analysis was performed using Access RT-PCR system (Promega) according to the manufacturer’s instructions. For RT-qPCR analysis, RNA was isolated using an RNeasy Plant Mini kit (Qiagen) and the real-time RT-PCR analysis was conducted using a Quantitect SYBR Green RT-PCR kit (Qiagen) in a CFX96TM real-time PCR detection system (Bio-Rad). Data
analysis and determination of reaction specificities were performed as described previously (Jeon et al., 2010). All real-time RT-PCR assays were conducted in duplicate for the same RNA isolated from each biological experiment. RT-qPCR analysis was performed for three different biological experiments and subjected to statistical analysis. Statistics were performed with SPSS21. Oligonucleotides and PCR conditions are provided in Supplemental Table 1.

Histochemical GUS Assays and Microscopy

Histochemical assays for GUS activity were performed with 5-bromo-4-chloro-3-indolyl glucuronide, as described previously (Jefferson and Wilton, 1991). For whole-mount visualization, the seedlings were cleared in 100% (v/v) ethanol for 24 h, and then mounted in 90% (v/v) glycerol. Samples were observed under a Leica DM2500 microscope at 50-, 200-, or 400-fold magnification with differential interference contrast or with a Nikon D300 Camera.

Transient Gene Expression Assays with Arabidopsis Protoplasts

In order to construct Pro35S:HA:ARR1 and Pro35S:L:3HA:EGFP effector plasmids, ARR1 or EGFP full-length DNA was first amplified by PCR using the Plt DNA polymerase and inserted into the Pro35S:GUS plasmid at Smal (N terminus) and ScaI (C terminus) sites in place of the GUS DNA fragment, yielding the Pro35S:ARR1 or Pro35S:EGFP DNA construct. A translational enhancer sequence (T) from the DR5(7X):GUS plasmid was then inserted into the Pro35S:HA(4X):ARR1 or the Pro35S:HA(3X):EGFP construct at BamHI (N terminus) and SpeI (C terminus) sites upstream of the translation initiation site, yielding the Pro35S:L:HA(4X):ARR1 or the Pro35S:L::HA(3X):EGFP DNA construct. Reporter plasmid was constructed by replacing GUS of Gal4(3X):GUS vector (Tiwarl et al., 2003) with the LUC DNA fragment. The Pro35S::L:CRF2 reporter construct was generated by replacing the Gal4(3X) DNA fragment of the Gal4(3X):LUC plasmid (Kang et al., 2013) with the CRF2 promoter encompassing the nucleotides from −2005 to −1 bp relative to the AUG initiation codon. These promoter regions were amplified by PCR from the genomic DNA of Arabidopsis Col-0 with primers harboring PstI site at the 5′-end and SpeI site at the 3′-end. The 35S::GUS vector was used as a transfection control (Lee et al., 2008). All the constructs were verified by DNA sequencing. The plasmids were purified using a Qiagen Plasmid Midi kit prior to protoplast transfection. Protoplasts were isolated from rosette leaves of 2- to 3-week-old Arabidopsis plants on an MS plate under a 16-h photoperiod, transfected with plasmid DNA, and incubated for 18 h in the dark at room temperature, as described previously (Lee et al., 2008). Total proteins were extracted using a lysis buffer (Promega) according to the manufacturer’s protocol. LUC activity was then determined using the Dual-Luciferase Reporter Assay System (Promega) with the Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio-TEK Instruments). LUC activity was normalized to the GUS activity. Transfection was performed in triplicate. Duplicate LUC and GUS assays were performed for each transfection. Oligonucleotides and PCR conditions are provided in Supplemental Table 1. Statistics were performed with SPSS21 using Student’s t test.

Preparation of the Recombinant Proteins

The ARR1 cDNA region coding for the DNA binding domain (1 to 300 amino acids) was amplified by PCR with gene-specific primers and inserted into the pGEX 4T-1 vector at BamHI (N terminus) and Xmal (C terminus) sites, yielding the GST:ARR1 plasmid. The recombinant proteins were produced in bacterial strain BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing the expression of recombinant proteins at 25°C overnight with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside in a shaking incubator. The cultured bacterial cells were lysed with 1× PBS buffer by sonication with the Vibra-Cell VCX130 (Sonics and Materials). The GST-fusion proteins were purified using glutathione-Sepharose 4B (GE Healthcare), according to the manufacturer’s instructions. The purified proteins were then dialyzed against the binding buffer (40 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 25% glycerol). Oligonucleotides and PCR conditions are provided in Supplemental Table 1.

EMSA

The EMSA was conducted essentially as described previously (Lee et al., 2013). To prepare DNA probes for EMSA, the oligonucleotides (29-mers) were denatured by boiling them for 5 min, followed by slow cooling to 23°C. The annealed oligonucleotides were radiolabeled with [α-32P]dCTP by the standard Klenow fill-in reactions and then purified on a G-50 micro columns (GE Healthcare), according to the manufacturer’s instructions. The binding reaction was performed in 10 μL of reaction mixture containing 400 fmol DNA probes, binding buffer (20 mM HEPES KOH, pH 7.6, 80 mM KCl, 1 mM DTT, and 10% glycerol), 200 ng of poly(dI-dC), 4 μg of BSA, and 250 ng of the purified GST fusion proteins at room temperature for 30 min. The samples were then analyzed by a 4.5% native polyacrylamide gel electrophoresis. Sequences for the DNA probes used in EMSA are provided in Supplemental Table 2.

ChiP Assays

The ChiP assays were conducted essentially as described previously (Jeon and Kim, 2011). Fifteen-day-old seedlings were treated with or without cold for 3 h. DNA from these seedlings was immunoprecipitated with an anti-c-Myc agarose affinity gel antibody (Sigma-Aldrich). Quantitative PCR analysis was conducted using SsoFast EvaGreen Supermix (Bio-Rad) on a CTF96 real-time system machine (Bio-Rad). The PCR primers for qPCR were designed to amplify the DNA fragments of 100 to 200 bp and are shown in Supplemental Table 1. The ACT717 DNA fragment was used for normalization.

Phenotypic Analysis

Phenotypic analysis was conducted as described previously (Park et al., 2002). Root length measurements were performed on five seeds from each line. The numbers of LRIs and LRP were scored using a Leica DM2500 microscope according to Malamy and Benfey (1997). LR induction experiments were conducted as described previously (Lee and Kim, 2013). Three-day-old seedlings were subjected to 90° gravitropic stimulus, and the numbers of LRPs at stages I-VIII (emerged) were determined at 30 and 54 h ppi. Statistics were performed with SPSS21, using Student’s t test or ANOVA with Tukey’s honestly significant difference test post-hoc analyses.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL database under the following accession numbers: CBF1 (At1g25490), CBF2 (At4g23750), CBF3 (At5g35290), AKH2 (At5g35750), AKH3 (At1g27320), AHP1 (At3g21510), AHP2 (At3g29350), AHP3 (At5g39340), AHP4 (At3g16360), AHP5 (At1g03430), ARR1 (At3g16857), ARR10 (At4g31920), ARR12 (At2g25180), ICE1 (At1g26774), ICE2 (At1g12860), and ACT717 (At5g09810). Sequence data used for hierarchical cluster analysis can be found under the following accession numbers: CBF1 (At4g11140), CBF5 (At2g45310), CBF6 (At3g16360), CBF7 (At1g22988), CPR9 (At1g71130), CPR10 (At1g68950), CPR11 (At5g25880), etc.
Supplemental Data

Supplemental Figure 1. Analysis of the Subcellular Distribution of EGFP:CRF2 and EGFP:CRF3 in Wild-Type Arabidopsis Mesophyll Protoplasts.

Supplemental Figure 2. RT-qPCR Analysis of ARR1 Expression in arr-1-3 Mutants and Pro35S:10xMYC:ARR1(ARR10X)arr-1-3 Transgenic Arabidopsis in Response to Cold.

Supplemental Figure 3. PCR Analysis of crf2-2, crf3-2, crf3-3, crf2-2 crf3-2, crf2-2 crf3-3 Mutants and Pro35S:3xHA:CRF2 and Pro35S:3xHA:CRF3 Transgenic Arabidopsis.

Supplemental Figure 4. Root Lengths and LR Densities of Wild-Type, crf3-2, and Pro35S:CRF3:3xHA/crf3-2 Transgenic Arabidopsis.

Supplemental Figure 5. Analysis of LRP Density at Stage I of Wild-Type, crf3-2 Mutants, and Pro35S:CRF3:3xHA/crf3-2 Transgenic Arabidopsis.

Supplemental Figure 6. Analysis of Cold Response of ProCRF2:EGFP:GUS and ProCRF3:EGFP:GUS Transgenic Arabidopsis.

Supplemental Figure 7. RT-qPCR Analysis of CRF2 and CRF3 Expression in ice1-2 and ice2 Mutant Backgrounds Compared with the Wild Type in Response to Cold.

Supplemental Figure 8. Hierarchical Cluster Analysis of CRF Genes in a Variety of Arabidopsis Mutants in Response to Phytohormones.

Supplemental Figure 9. ANOVA Tables for Analyses Reported in Figures 1C, 2, 3A, 3C, 5B, 5C, 6B, 7C, and 7D and Supplemental Figures 4B, 4C, and 5.

Supplemental Table 1. Oligonucleotides and PCR Conditions.

Supplemental Table 2. Sequences for the DNA Probes Used in EMSA

ACKNOWLEDGMENTS

We thank T. Aoyama for providing us with the 3SS:ARR1∆DDK:GR seeds, T. Nakagawa for the pGWB vectors, and the ABRC for the T-DNA insertion mutants. This study was supported by grants to J.K. from the Next-Generation BioGreen21 Program (PJ01104701), Rural Development Administration, Republic of Korea, the Basic Science Research Program (2010-0022850) through the National Research Foundation of Korea, funded by the Ministry of Education, Science, and Technology of Korea, and Chonnam National University, 2013.

AUTHOR CONTRIBUTIONS

J.J., C.C., M.R.L., and N.V.B. designed and conducted the experiments and analyzed the data. J.J. wrote the article. J.K. conceived the project, designed the experiments, analyzed the data, and wrote and edited the article.

Received October 23, 2015; revised May 24, 2016; accepted July 14, 2016; published July 18, 2016.

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CYTOKININ RESPONSE FACTOR2 (CRF2) and CRF3 Regulate Lateral Root Development in Response to Cold Stress in Arabidopsis
Jin Jeon, Chuloh Cho, Mi Rha Lee, Nguyen Van Binh and Jungmook Kim
Plant Cell 2016;28;1828-1843; originally published online July 18, 2016;
DOI 10.1105/tpc.15.00909

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