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A Subset of Cytokinin Two-component Signaling System Plays a Role in Cold Temperature Stress Response in *Arabidopsis*[*][S]

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A multistep two-component signaling system is established as a key element of cytokinin signaling in *Arabidopsis.* Here, we provide evidence for a function of the two-component signaling system in cold stress response in *Arabidopsis*. Cold significantly induced the expression of a subset of A-type *ARR* genes and of *GUS* in Pro_{ARR7}:*GUS* transgenic *Arabidopsis*. *AHK2* and *AHK3* were found to be primarily involved in mediating cold to express A-type *ARR*s despite cytokinin deficiency. Cold neither significantly induced *AHK2* and *AHK3* expression nor altered the cytokinin contents of wild type within the 4 h during which the A-type *ARR* genes exhibited peak expression in response to cold, indicating that cold might induce *ARR* expression via the AHK2 and AHK3 proteins without alterations in cytokinin levels. The *ahk2 ahk3* and *ahk3 ahk4* mutants exhibited enhanced freezing tolerance compared with wild type. These *ahk* double mutants acclimated as efficiently to cold as did wild type. The overexpression of the cold-inducible *ARR7* in *Arabidopsis* resulted in a hypersensitivity response to freezing temperatures under cold-acclimated conditions. The expression of C-repeat/dehydration-responsive element target genes was not affected by *ARR7* overexpression as well as in *ahk* double mutants. By contrast, the *arr7* mutants showed increased freezing tolerance. The *ahk2 ahk3* and *arr7* mutants showed hypersensitive response to abscisic acid (ABA) for germination, whereas *ARR7* overexpression lines exhibited insensitive response to ABA. These results suggest that *AHK2* and *AHK3* and the cold-inducible A-type *ARR*s play a negative regulatory role in cold stress signaling via inhibition of ABA response, occurring independently of the cold acclimation pathway.

Cytokinins are plant hormones that regulate a variety of developmental and physiological processes, including cell division, cell proliferation, root and leaf differentiation, chloroplast biogenesis, and the inhibition of leaf senescence (1). *Arabidopsis* cytokinin signaling utilizes a multistep phospho-relay composed of a sensor kinase, a histidine phosphotransfer protein, and a response regulator similar to the TCS[2] of bacterial and yeast cells (2). A hybrid-type histidine kinase referred to as CYTOKININ INDEPENDENT1 (CKI1) is essential for megagametogenesis (3). CYTOKININ RESPONSE1 (CRE1)/WOODEN LEG1 (WOL1)/ARABIDOPSIS HISTIDINE KINASE4 (AHK4) were shown to bind directly to a variety of natural and synthetic cytokinins *in vitro* with high specificity as well as in a yeast system and thus to be a primary receptor for cytokinins (4–8). The experiments conducted using a heterologous phospho-relay system demonstrated that AHK2 and AHK3 are also cytokinin receptors. The primary functions of these *Arabidopsis* histidine kinase (*AHK*) genes involve the triggering of cell division and the maintenance of the meristematic competence of cells to prevent subsequent differentiation (9, 10). Partially redundant functions of cytokinin receptors have also been revealed in shoot growth, root development, leaf senescence, seed size, germination, and cytokinin metabolism (11). The AHK2 and AHK3 receptors play prominent roles in the control of leaf development, whereas AHK4 functions in the roots (10). AHK3 is also involved in the cytokinin-mediated control of leaf longevity (12). AHK1 has been shown to function as an osmosensor in yeasts in a cytokinin-independent manner (13), and it has recently been demonstrated to be a positive regulator of drought and salt stress response and ABA signaling (14). AHK5 (CKI2) has histidine kinase activity independent of cytokinins and functions as a negative regulator for root elongation via an ETR1-dependent ABA and ethylene signaling pathway (15). AHK5 was found to integrate endogenous and environmental signals that generate H_2O_2 in guard cells (16). A recent study showed that the cytokinin-independent activity of CKI1 and cytokinin-induced AHK2 and AHK3 are important for vascular bundle formation in *Arabidopsis* (17).

The *Arabidopsis* genome harbors six genes encoding histidine phosphotransfer proteins (AHPs) that mediate the transfer of the phosphoryl group from the histidine protein kinases

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Figs. 1–8.

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[2] The abbreviations used are: TCS, two-component signaling system; ABA, abscisic acid; CBF/DREB, C-repeat-binding factor/dehydration-responsive element-binding factor; DEX, dexamethasone; BA, benzyladenine; GUS, β-glucuronidase; Mes, 4-morpholineethanesulfonic acid; RT, reverse transcription; MS, Murashige-Skoog; ARR, *Arabidopsis* response regulator; AHK, *Arabidopsis* histidine kinase.

to the response regulators (18). Most of the *Arabidopsis* histidine phosphotransfer proteins act as redundant, positive regulators of cytokinin signaling, except for AHP4 and AHP6, and affect many aspects of plant development (19, 20). There are 32 genes encoding 23 putative response regulators and 9 pseudo-response regulators in *Arabidopsis*. The typical response regulators are classified into either type A or B (18, 21). The type B *Arabidopsis* response regulators (ARRs) are transcription factors that contain a receiver domain and a large C-terminal region harboring a Myb-like DNA-binding domain and a glutamine-rich domain and are not cytokinin-inducible. The middle segments of ARR1 and ARR2, the B-type ARRs, can bind to DNA in a sequence-specific manner *in vitro*, and their C-terminal halves function as transactivation domains in plant cells (22). ARR1, ARR2, and ARR10 have been demonstrated to function as transcriptional activators for the *ARR6* promoter in *Arabidopsis* mesophyll cell protoplasts (23). Type B ARRs function as positive regulators of cytokinin signaling and also play a role in plant development (24, 25). By contrast, the typical type A ARRs are composed of a receiver domain and a divergent C-terminal extension. The mRNAs of A-type *ARR*s accumulate rapidly and transiently after cytokinin treatment. The A-type ARRs function as transcriptional repressors in *Arabidopsis* protoplasts (23). In transgenic *Arabidopsis*, *ARR8* or *ARR15* overexpression resulted in reduced cytokinin sensitivity, whereas *ARR4* overexpression induced increased sensitivity to exogenous cytokinins (26, 27). The majority of A-type ARRs are partially redundant negative regulators of cytokinin signaling (28). The genome-wide expression profiling analysis of cytokinin response in the *ARR7* overexpressor compared with the wild type demonstrated that ARR7 acts principally as a transcriptional repressor of a variety of early cytokinin-regulated genes encoding transcription factors, signal transmitters, plant development, and cellular metabolism (29). The Asp-85 mutation of ARR7 into the Asp residue blocked the phosphorylation of ARR7 by the *Arabidopsis* protein extracts *in vitro* and abolished the ability of ARR7 to suppress cytokinin-mediated responses in transgenic *Arabidopsis* (30), suggesting that the phosphorylation of ARR7 is essential to its function as a negative regulator of cytokinin signaling in *Arabidopsis*.

Recent studies have shown that AHK1 is a positive regulator of drought and salt stress responses, as well as ABA signaling (14), and is involved in water stress response during the early vegetative stages of plant growth, but it also performs a unique role in the regulation of desiccation processes during seed maturation (31). AHK2, AHK3, and CRE1 have all been implicated as negative regulators in ABA signaling and osmotic stress responses (14).

Cold temperatures exert adverse effects on plant growth and development and limit the geographic distribution of plants. Plants respond and adapt to cold via alterations in various aspects of physiological processes, including gene expression (32, 33). Abiotic stresses, including cold, induce a number of genes that encode the proteins that promote stress tolerance, mediated primarily by an ABA-dependent and/or ABA-independent pathway. Cold temperatures transcriptionally activate C-REPEAT-BINDING FACTOR/DRE-BINDING FACTORs (CBF/DREBs) that bind to the C-repeat/dehydration-responsive element in the ABA-independent pathway, inducing the expression of cold-responsive genes. Genetic and reverse genetic approaches have revealed a number of components that are involved in the cold signaling pathway (32, 34–36).

In this study, we analyzed *ahk* and A-type *arr Arabidopsis* mutants as well as *ARR7*-overexpressing lines under freezing temperatures and A-type *ARR* expression in response to cold, and we demonstrated that the TCS, which is known best as a mediator of cytokinin signal transduction, is employed as a negative regulator in the process of adaptation to cold temperatures in plants. We found that the cold-inducible expression of A-type *ARR* genes was severely reduced by *ahk2* and *ahk3* double mutations. Analyses of A-type *ARR* expression in *ahk* mutants and in cytokinin-deficient or -overproducing transgenic *Arabidopsis* indicate that *AHK2* and *AHK3* might be involved in mediating cold to express A-type *ARR*s independently of the influence of endogenous cytokinin levels. Moreover, the cytokinin contents were not altered significantly by cold treatment within the 4 h during which the peak expression of cold-inducible *ARR*s occurs. Cold did not significantly induce *AHK2* and *AHK3* expression, indicating that the AHK2 and AHK3 proteins may mediate cold temperatures for A-type *ARR* expression. The *ahk2*, *ahk3*, and *cre1* (*ahk4*) single mutants displayed tolerance to freezing temperatures when preincubated with cytokinin prior to freezing, as compared with the wild-type plants treated with cytokinin, as well as the untreated *ahk* single mutants. The *ahk2 ahk3* and *ahk3 ahk4* double mutants exhibited enhanced freezing tolerance without cytokinin preincubation. The overexpression of the cold-inducible *ARR7* in *Arabidopsis* caused a hypersensitivity response to freezing temperatures when cold-acclimated, whereas the *arr7* mutants exhibited increased freezing tolerance prior to and after cold acclimation. The expression of CBF/DREB target genes was not affected by *ARR7* overexpression as well as in *ahk* double mutants. In both root elongation and germination assays, *ahk2 ahk3* showed hypersensitivity response to ABA. *arr7* showed hypersensitive response to ABA for germination, whereas Pro_{35S}:*ARR7* lines exhibited insensitive response to ABA. These results suggest that a normal function of a part of cytokinin TCS might be to negatively regulate the cold stress adaptation response through inhibition of ABA response, independently of the CBF/DREB pathway. Our results reveal a new versatile function of the plant two-component signaling system in cold stress response.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Arabidopsis Transformation—The promoter region of *ARR7* encompassing −1651 bp relative to the AUG initiation codon was isolated by PCR from the genomic DNA of *Arabidopsis* Col-0 and subcloned into pBI121 (Clontech) in place of the CaMV 35S promoter, and transgenic *Arabidopsis* containing this construct (Pro_{ARR7}:*GUS*) was made by *Agrobacterium*-mediated transformation (37). T3 homozygous transformants were prepared and amplified. All constructs were verified by DNA sequencing prior to plant transformation.

ahk and arr Mutants ahk2-1, *ahk3-1*, *ahk2-1 ahk3-1* were generously provided by Dr. Ueguchi and confirmed via geno-

typing prior to usage (7, 10). *cre1-12*, *ahk2-2 ahk3-3*, *ahk2-2 cre1-12*, and *ahk3-3 cre1-12* were kindly provided by Dr. Kakimoto (9) and verified by genotyping prior to usage. *arr5* (CS25267), *arr6* (CS25268) (28), and *arr7* were obtained from ABRC. *arr7* was WiscDsLox T-DNA lines (supplemental Fig. 1). The mutants were verified by genotyping prior to usage.

Transgenic Arabidopsis Harboring 35S:AtCKX2-2 (Arabidopsis thaliana Cytokinin Oxidase/Dehydrogenase2-2), LhGR-N, or pV-ipt/LhGR-N Constructs—*35S:AtCKX2-2* seeds were kindly provided by Dr. Schmülling (38) and *LhGR-N*(4c-S5) and *pV-ipt/LhGR-N*(ipt5-2) seeds were generously provided by Dr. Moore (39). The transgenic seeds were confirmed by genotyping prior to amplification and usage. The PCR conditions and primer sequences are provided in supplemental Table 1.

Cold, Hormone, and DEX Treatment—*A. thaliana* was grown on germination agar plates containing 0.5× Murashige-Skoog (MS) media with vitamins, 1.5% sucrose, 2.5 mM Mes, pH 5.7, and 0.8% agar at 23 °C with a 16-h photoperiod and treated essentially as described previously (40). For cold treatment, the light-grown seedlings were incubated at 1 °C with white fluorescent light. For treatment with cytokinin benzyladenine (BA), the seedlings were grown on sterile filter papers on MS agar plates and then immersed in MS medium containing 5 μM BA. For the freezing tolerance assays, the plants were germinated on MS agar plates, grown for the indicated periods, and then treated with freezing temperatures in a temperature-controlled chamber. For the DEX treatments, the plants were grown on sterile filter paper on MS plates for 10 days and then transferred to MS plates containing 10 μM DEX. The plants were then incubated for a given period.

Electrolyte Leakage Test—Freezing-induced electrolyte leakage (41) was determined from the rosette leaves of 2-week-old plants grown in soil or in MS agar plates. The excised leaflets were placed individually into 5-ml test tubes containing 100 μl of deionized water in a refrigerated circulator bath (Polysciences) at 0 °C, and the temperature of the bath was programmed to decrease to −10 °C at 1 °C decrements over 30 min. The percentage of electrolyte leakage was calculated as the percentage of the conductivity prior to autoclaving over that noted after autoclaving the leaflets. At least 8–10 assays were conducted for each sample.

Histochemical GUS Assays—Histochemical assays of GUS activity were conducted after 24 h of incubation with the treated seedlings in 5-bromo-4-chloro-3-indolyl glucuronide (Duchefa, The Netherlands) at 37 °C and removal of the chlorophyll from green tissues by incubation in 100% ethanol, as described previously (42).

Cytokinin Analysis—The procedure utilized for cytokinin purification herein was a modified version of the method described previously (43). Deuterium-labeled cytokinin internal standards (Olchemim Ltd., Czech Republic) were added, each at 1 pmol per sample, to assess the recovery during purification and to validate the determination (44). The samples were purified with a combined cation (SCX cartridge) and anion (DEAE-Sephadex C18 cartridge) exchanger and immunoaffinity chromatography based on wide range-specific monoclonal antibodies against cytokinins (45). The metabolic eluates from the immunoaffinity chromatography columns were evaporated to dryness and dissolved in 20 μl of the mobile phase utilized for quantitative analysis. The samples were analyzed by an ultra-performance liquid chromatography (Acquity UPLC™; Waters, Milford, MA) coupled to a Quatro *micro*™ atmospheric pressure ionization (Waters, Milford, MA) triple quadrupole mass spectrometer equipped with an electrospray interface. The purified samples were then injected into a C18 reversed-phase column (BEH C18; 1.7 μm; 2.1 × 50 mm; Waters). The column was eluted with a linear gradient (0 min, 10% B; 0–8 min, 50% B; flow-rate of 0.25 ml/min; column temperature of 40 °C) of 15 mM ammonium formate, pH 4.0 (solvent A), and methanol (solvent B). Quantification was conducted by the multiple reaction monitoring of $[M + H]^+$ and the appropriate product ion. For the selective multiple reaction monitoring experiments, the optimal conditions, dwell time, cone voltage, and collision energy in the collision cell corresponding to exact diagnostic transition were optimized for each cytokinin (44). Quantification was conducted using Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to the appropriate labeled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to a known quantity of an added internal standard (45).

RNA Isolation, RT-PCR, and RNA-Gel Blot Analysis—Following treatment, the *Arabidopsis* plants were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from frozen *Arabidopsis* samples using TRI Reagent® (Molecular Research Center, Inc.). Total RNA was separated on 1.2% agarose gel, transferred to nylon membranes, hybridized with ^{32}P-labeled DNA probes at 68 °C for 3 h using 10 ml of QuikHyb solution (Stratagene), and then washed. The blot was subsequently exposed to x-ray film. For RT-PCR analysis, total RNA was isolated using the RNeasy plant mini kit (Qiagen) and subjected to RT-PCR analysis with an Access RT-PCR System (Promega) according to the manufacturer's instructions. RT-PCR conditions and primer sequences are provided in supplemental Table 1.

Real Time RT-PCR—Real time RT-PCR was conducted using a QuantiTect SYBR Green RT-PCR kit (Qiagen) in a Rotor-Gene 2000 real time thermal cycling system (Corbett Research). PCR conditions and primers are available upon request. To determine the copy numbers of the transcripts in the treated samples, real time PCR was conducted for each sample with a known quantity of the *in vitro* transcribed RNA (Promega), yielding specific threshold values (C_t). A standard curve was generated to show the linear correlation between the log (copy numbers of the RNA) and the C_t. The copy numbers of the transcripts of unknown samples subjected to real time RT-PCR were then calculated from this standard curve. Real time RT-PCR conditions and primer sequences are provided in supplemental Table 1.

RESULTS

ARR7 and Pro_{ARR7}:GUS Respond to Cold Temperature with Expression Profiling Similar to Cytokinin Response—Many of the upstream components of signaling pathways are expressed

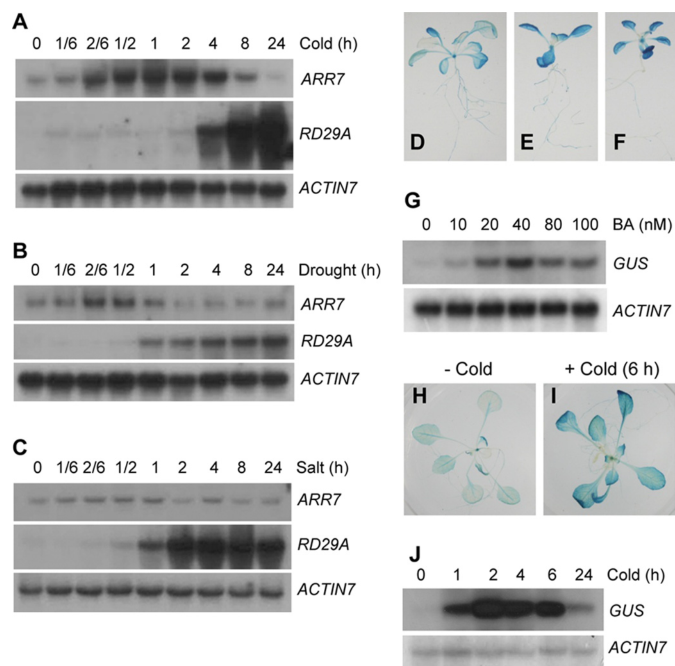

FIGURE 1. ***ARR7* and *Pro$_{ARR7}$:GUS* transgenics respond to abiotic stresses, including cold.** *A*, response of *ARR7* to cold. Eleven-day-old wild-type (Col-0) seedlings were incubated at 1 °C. Total RNA isolated from each treatment was subjected to RNA-gel blot analysis. *RD29A* was used as a marker gene (62). *ACTIN7* was utilized as a loading control. *B*, response of *ARR7* to dehydration. Three-week-old wild-type seedlings were dehydrated on filter paper in the light at 23 °C and analyzed as described in *A*. *C*, response of *ARR7* to high salinity. Eleven-day-old wild-type seedlings were incubated with 300 mM NaCl in darkness and analyzed as described in *A*. *D–G*, response of *Pro$_{ARR7}$:GUS* to BA. Plants grown for 16 days on MS agar plate containing 0 nM (*D*), 40 nM (*E*), and 100 nM (*F*) BA were subjected to histochemical GUS assays. Plants grown for 16 days on MS agar plates containing varying concentrations of BA were subjected to RNA-gel blot analysis (*G*). *H–J*, response of *Pro$_{ARR7}$:GUS* to cold. *Pro$_{ARR7}$:GUS* seedlings (17-day-old light-grown plants) grown on MS agar plates were treated with cold at 1 °C for 6 h and subjected to histochemical GUS assays (*H* and *I*) or treated with cold at 1 °C for the indicated time, followed by RNA-gel blot analysis (*J*).

quickly and primarily in response to stimuli (46, 47). We previously isolated *ARR7*, a cytokinin-inducible A-type *ARR* gene (48), as an immediate response mRNA from *Arabidopsis* by cold temperature at 1 °C using differential display PCR.[3] We thus investigated the *ARR7* gene as a starting point to understand the potential cross-talk occurring between cold response and cytokinin signaling and the role of TCS in cold response. We initially performed a time course analysis of the expression of *ARR7* mRNA in response to cold via RNA-gel blot analysis (Fig. 1*A*). The *ARR7* mRNA began to accumulate within 10 min at 1 °C, reached a peak level at 2–4 h, and then declined, displaying an early and transient expression pattern similar to that observed with cytokinin-mediated *ARR7* expression. *ARR7* also responds to other abiotic stresses, such as drought (Fig. 1*B*) or significantly high salinity (Fig. 1*C* and supplemental Fig. 2), but only slightly and in a transient manner, with an earlier peak. In this study, we have focused on the role of TCS in cold response. To determine whether *ARR7* expression is modulated at the transcriptional level in response to cold as well as cytokinins, we fused the 1.65-kbp promoter region of the *ARR7* gene to *GUS* and generated six transgenic *Arabidopsis* lines harboring the

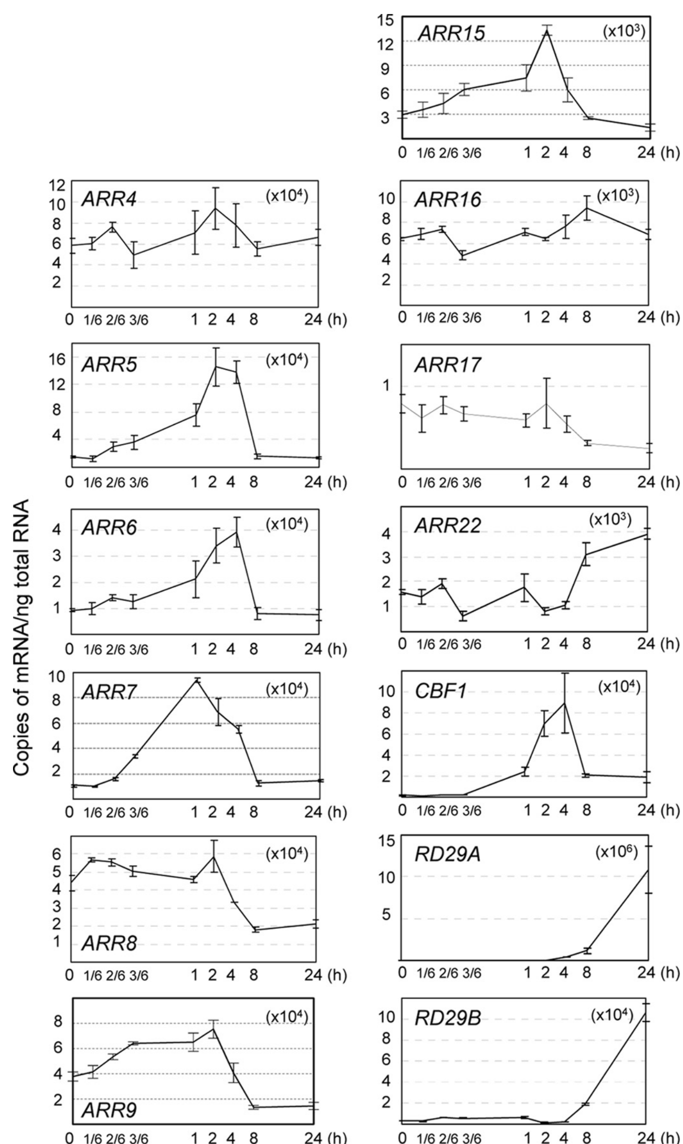

FIGURE 2. **Quantitative analysis of A-type *ARR* gene expression in response to cold.** Eleven-day-old wild-type (Col-0) seedlings were incubated at 1 °C for varying periods of time (hour). Total RNA isolated from each treatment was subjected to quantitative real time RT-PCR. *CBF1*, *RD29A*, and *RD29B* were employed to verify stress-inducible gene expression. *ACTIN7* was utilized as a loading control. Copies of the transcripts from cold-treated plants were plotted per ng of total RNA after normalization to *ACTIN7* RNA. Multiplication of the *number* in parentheses in the *graph* by the *number* in the *y* axis generates copy numbers of the corresponding transcripts. The means ± S.E. from biological triplicate experiments were plotted.

corresponding construct (*Pro$_{ARR7}$:GUS*). We conducted histochemical GUS assays to assess the patterns of tissue expression. As shown in supplemental Fig. 3, *A–G*, we detected strong GUS expression in the meristem regions of the flowers, shoots, and roots (supplemental Fig. 3, *A–C*). The vascular tissues of the leaves and roots displayed strong GUS staining (supplemental Fig. 3, *D, E,* and *G*). The valves of the silique (supplemental Fig. 3*F*) and the pistil tip (supplemental Fig. 3*A*) were also strongly stained with GUS. Increasing concentrations of cytokinin benzyladenine (BA) enhanced GUS staining throughout the whole leaves (Fig. 1, *D–F*) and the *GUS* mRNA level, as demonstrated by RNA-gel blot analysis (Fig. 1*G*). Cold temperature significantly

[3] M.-O. Oh and J. Kim, unpublished observations.

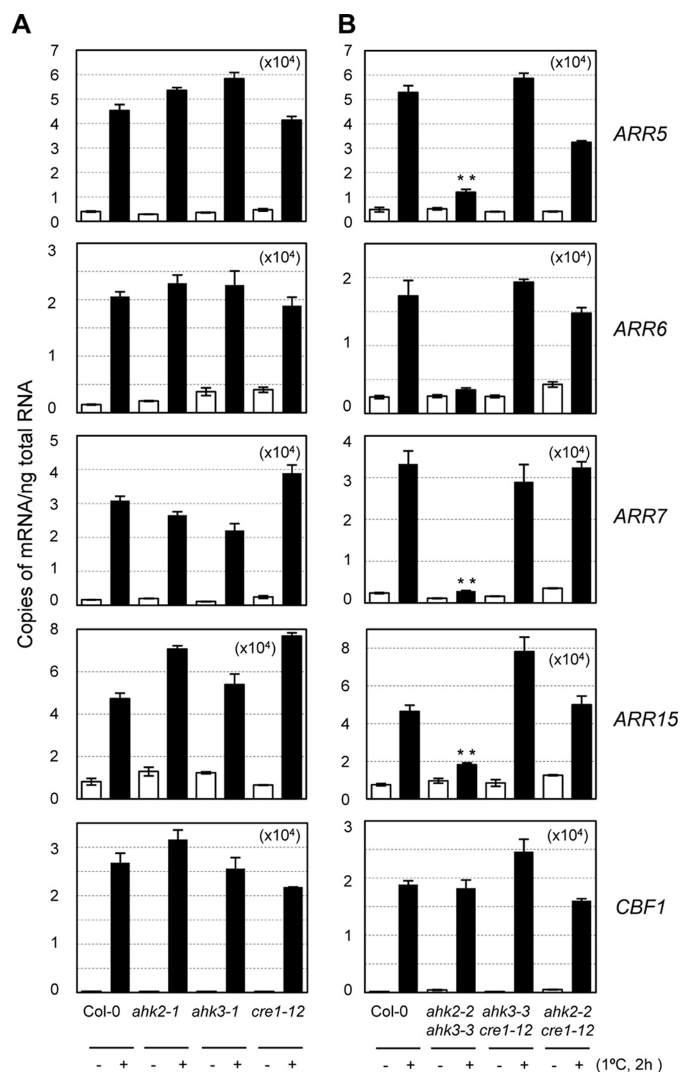

FIGURE 3. **Expression of *ARR5, ARR6, ARR7*, and *ARR15* in response to cold in *ahk* mutants compared with the wild-type plants.** *A, ARR5*, *ARR6*, *ARR7*, and *ARR15* expression in *ahk* single mutant backgrounds. Eleven-day-old light-grown seedlings were treated for 2 h at 1 °C, and total RNA isolated was subjected to real time RT-PCR. Treatment and analysis of the samples were done as described in the Fig. 2 legend. *Closed* and *open bars* represent the transcript levels from the plants treated with or without cold, respectively. *B, ARR5*, *ARR6*, *ARR7*, and *ARR15* expression in *ahk* double mutant backgrounds. Treatment and analysis of the samples were done as described in *A*. ** denotes statistically significant changes with $p < 0.01$ as compared with the samples without cold treatment.

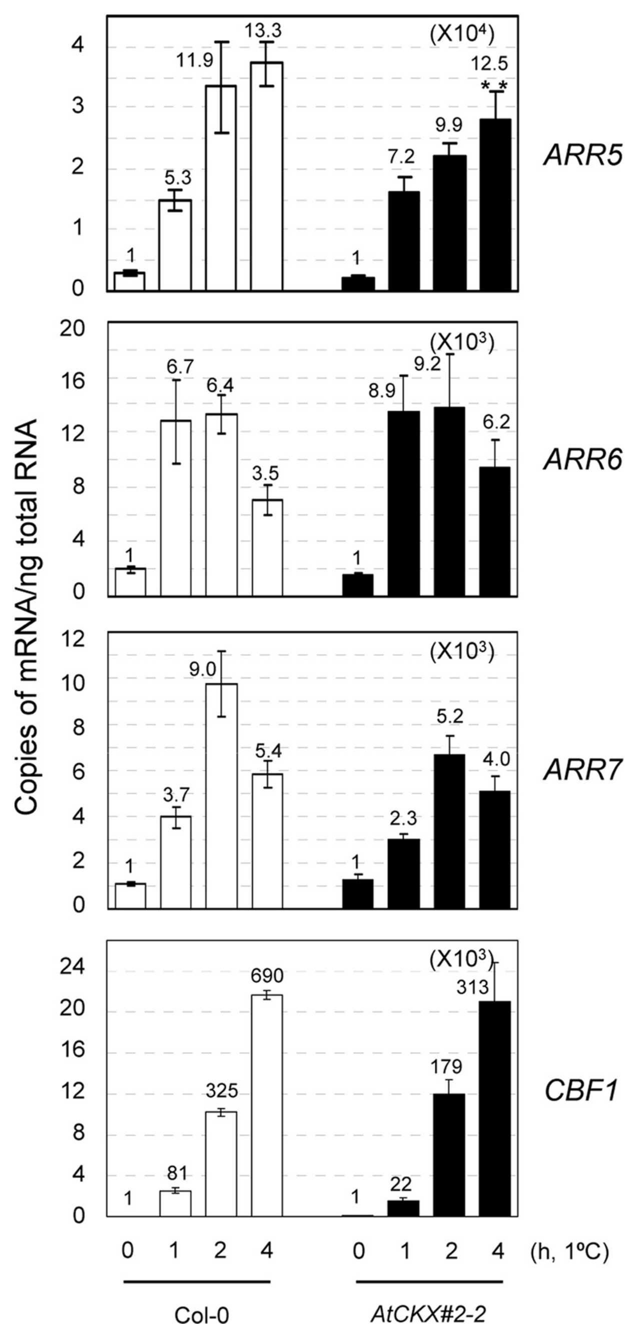

FIGURE 4. **Expression of *ARR5, ARR6,* and *ARR7* in response to cold in *35S*: *AtCKX* transgenic *Arabidopsis* deficient in cytokinins compared with the wild type.** Eleven-day-old light-grown seedlings were treated at 1 °C for 1, 2, or 4 h, and the total RNA isolated was subjected to real time RT-PCR. The treatment and analysis of the samples were done as described in the Fig. 2 legend. The *numbers* on top of the *bars* indicate fold-induction relative to the control sample at 0 h. *Open* and *closed bars* represent the Col-0 wild type and *35S:AtCKX2-2* transgenic *Arabidopsis*, respectively. ** denotes statistically significant changes with $p < 0.01$ compared with the wild type.

enhanced GUS expression (Fig. 1, *H* and *I*). The RNA-gel blot analysis also demonstrated that cold temperature transiently induced the levels of the *GUS* mRNA (Fig. 1*J*). We observed relatively stronger induction of the *GUS* mRNA as compared with GUS staining in response to cold temperatures. This may be attributable to the strong background of GUS staining prior to cold treatment, because of the stable GUS enzymes. These data demonstrate that exposure to cold as well as cytokinin activates *ARR7* transcription. Our observations regarding the tissue-specific expression patterns of GUS and the timing of cold-responsive *ARR7* expression are generally consistent with the microarray data as visualized by the *Arabidopsis* electronic fluorescent pictograph browser (49).

Cold Temperature Induces Expression of a Subset of A-type ARR Genes—We next assessed the response of all A-type *ARR* genes (18, 21) to cold to determine whether or not cold-induced *ARR7* expression is unique among A-type *ARR* genes. Both comparative RT-PCR and quantitative real time RT-PCR assays were employed and demonstrated that cold temperatures transiently induced the expression of a variety of A-type *ARR* genes, including *ARR5*, *ARR6*, *ARR7*, and *ARR15* (Fig. 2). *ARR9* and

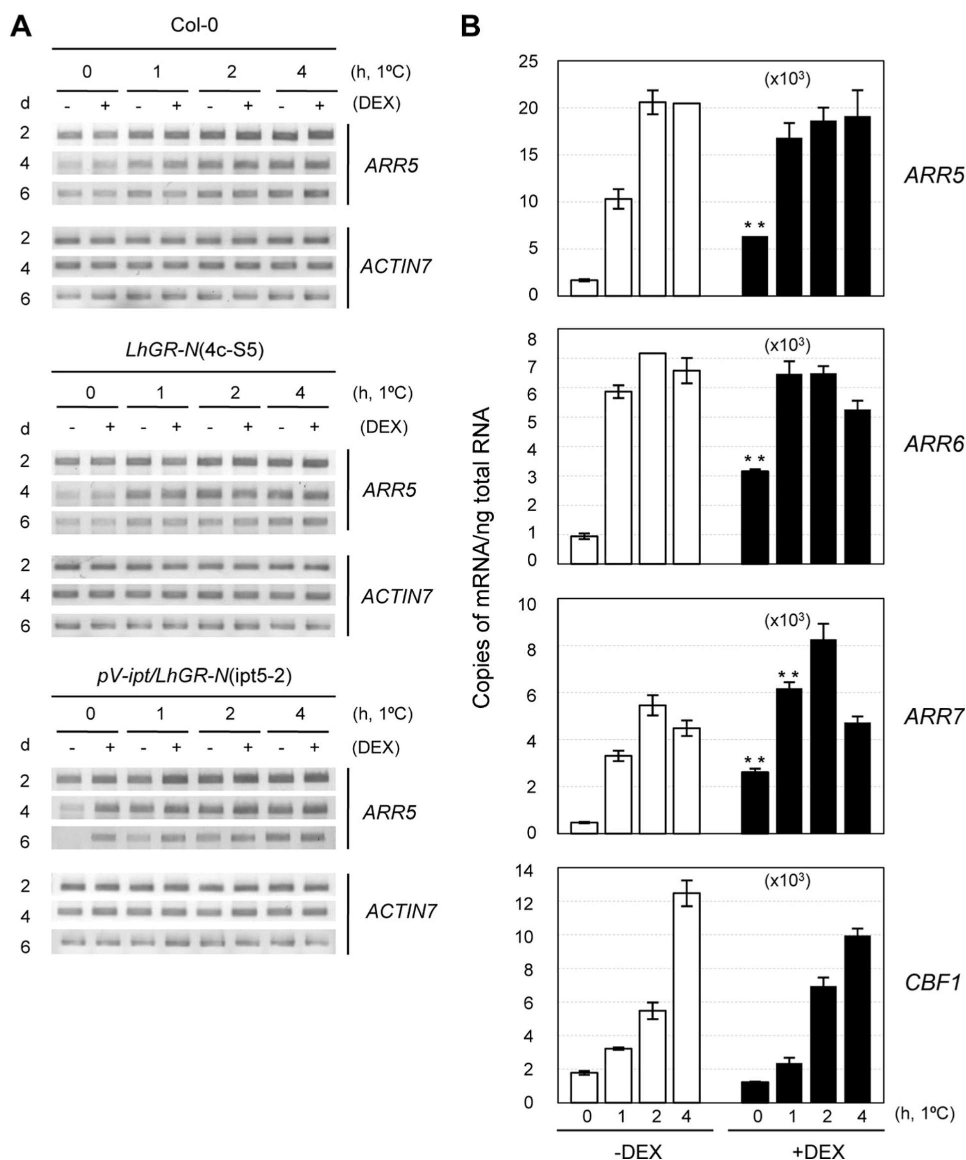

FIGURE 5. **Effects of cytokinin overproduction on cold-responsive expression of *ARR5, ARR6*, and *ARR7*.** *A*, RT-PCR analysis of cold-responsive *ARR5* expression with or without cytokinin overproduction. The *pV-ipt/LhGR-N*(ipt5-2) transgenic plants as well as control plants, the wild-type, and *LhGR-N*(4c-S5) plants were grown on sterile filter paper on MS plates for 10 days and then incubated on MS plates with or without 10 μM DEX for an additional 2, 4, or 6 days in the light. The plants were then treated with cold at 1 °C in the light for the indicated number of hours. Total RNA was isolated from treated plants and subjected to RT-PCR for *ARR5*. *ACTIN7* was utilized as a loading control. Inverted images of the RT-PCR products are shown. *LhGR-N* transgenic plants contain activator construct expressing *LhGR* from the CaMV 35S promoter (39). *pV-ipt/LhGR-N* transgenic plants contain the activator construct expressing *LhGR* and a construct harboring *IPT* and *GUS* under six copies of an ideal *lac* operator (39). 4c-S5 and ipt5-2 indicate the line numbers. *B*, real time RT-PCR analysis of cold-responsive *ARR* expression with or without cytokinin overproduction. Plants were treated as described in *A* and analyzed by real time RT-PCR for *ARR5*, *ARR6*, and *ARR7*, as described in the Fig. 2 legend. *CBF1* was utilized to verify cold-inducible gene expression. ** denotes statistically significant changes with $p < 0.01$ compared with the absence of DEX.

ARR22 showed a weak and early or late response to cold, respectively. Similar patterns were noted on comparative RT-PCR (data not shown). Although the quantification of the *ARR3* mRNA could not be clearly determined due to the double bands of the PCR products, the RT-PCR data showed that *ARR3* did not respond significantly to cold (data not shown). Our results are consistent with previous microarray analysis data demonstrating that *ARR5* and *ARR7* exhibited maximum cold response in 2–4 h with induction levels over 8- and 5-fold, respectively (50). *CBF1*, *RD29A*, and *RD29B* were utilized as cold marker genes to confirm the effects of our cold treatment with regard to the induction of gene expression. *CBF1* was unresponsive to cytokinin BA, although BA greatly induced *ARR7* expression (supplemental Fig. 4).

Double Mutations in AHK2 and AHK3 Severely Reduced ARR5, ARR6, ARR7, and ARR15 Expression in Response to Cold but Do Not Affect the Cold-induced Expression of CBFs—The observation that *ARR7* promoter-*GUS* and various A-type *ARR* genes responded to cold raised the possibility that the *AHK* genes encoding cytokinin receptors might be involved in mediating cold signals for the activation of A-type *ARR* expression. To evaluate this possibility, we analyzed the expression of a representative set of cold-inducible A-type *ARR* genes, *ARR5*, *ARR6*, *ARR7*, and *ARR15* in *ahk2*, *ahk3*, and *cre1* (=*ahk4*) mutant backgrounds in response to cold by using both quantitative real time and comparative RT-PCR methods. All *ahk* mutants were T-DNA insertional mutants with the Col-0 ecotype (9, 10). Plants were treated with cold for the peak expression time of 2 h. In all single *ahk* mutants, *ahk2-1*, *ahk3-1*, and *cre1-12*, *ARR5*, *ARR6*, *ARR7*, and *ARR15* showed wild-type levels of gene expression in response to cold (Fig. 3*A*). In contrast, the *ahk2 ahk3* double mutants, *ahk2-2 ahk3-3*, exhibited severely reduced transcript levels of *ARR5*, *-6*, *-7*, and *-15* to cold, whereas the other *ahk* double mutants, *ahk3-3 cre1-12* and *ahk2-2 cre1-12*, showed a normal response to cold (Fig. 3*B*). Similar patterns were noted on comparative RT-PCR (data not shown). The *ahk2-1 ahk3-1* double mutants also exhibited similarly reduced transcript levels (data not shown). These findings indicate that *AHK2* and *AHK3* perform a primary role in mediating cold temperatures for the expression of A-type *ARR*s, whereas other *AHK*s not related to cytokinin reception or other mechanisms may also be involved. In these *ahk* mutants, *CBF1*, encoding the critical transcription factor that functions in the cold acclimation process, can be induced by cold treatment as efficiently as in the wild-type plants. Other *CBF* members, *CBF2* and *CBF3*, also responded to cold temperatures in *ahk* double mutant backgrounds, similarly to the wild-

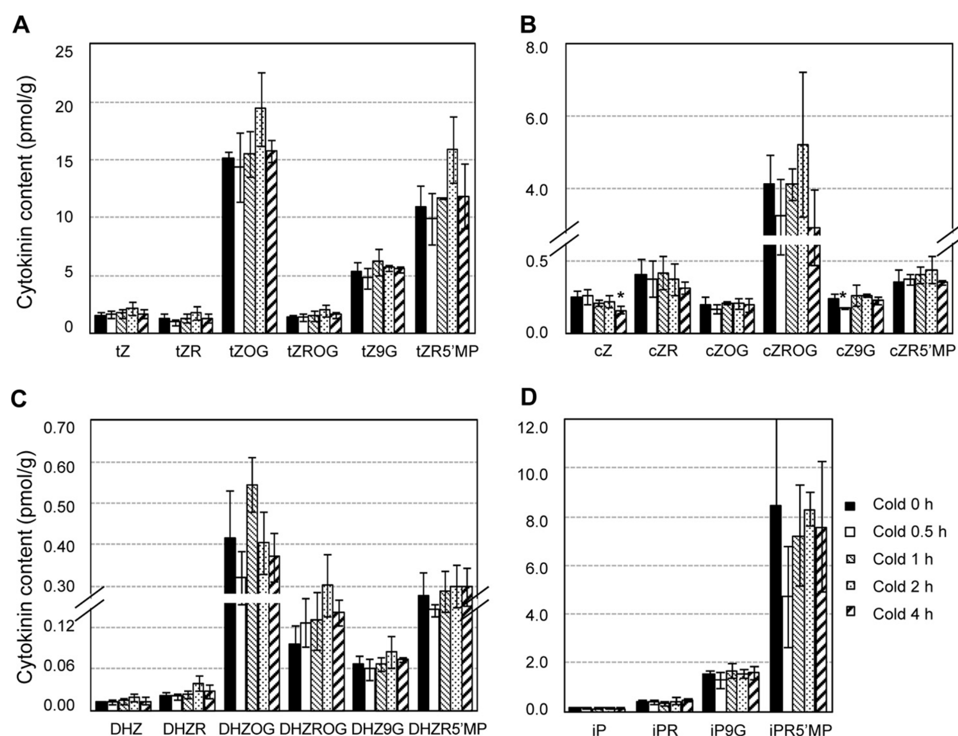

FIGURE 6. **Effects of cold on cytokinin content in the wild-type *Arabidopsis* plants.** *A,* content of various forms of *trans*-zeatin. One gram of 10-day-old light-grown *Arabidopsis* Col-0 seedlings (10 days after germination) per sample was pooled, and three independent biological samples were obtained for each treatment of cold temperature at 1 °C for a given period of time. The data shown are the means (pmol/g fresh weight) ± S.D. * denotes statistically significant changes with $p < 0.05$ compared with the wild type. *tZ, trans*-zeatin; *tZR, trans*-zeatin riboside; *tZOG, trans*-zeatin *O*-glucoside; *tZROG, trans*-zeatin riboside *O*-glucoside; *tZ9G, trans*-zeatin 9-glucoside; *tZR5′MP, trans*-zeatin riboside 5′-monophosphate; *cZ, cis*-zeatin; *cZR, cis*-zeatin riboside; *cZOG, cis*-zeatin *O*-glucoside; *cZROG, cis*-zeatin riboside *O*-glucoside; *cZ9G, cis*-zeatin 9-glucoside; *cZR5′MP, cis*-zeatin riboside 5′-monophosphate; *DHZ*, dihydrozeatin; *DHZR*, dihydrozeatin riboside; *DHZOG*, dihydrozeatin *O*-glucoside; *DHZROG*, dihydrozeatin riboside *O*-glucoside; *DHZ9G*, dihydrozeatin 9-glucoside; DHZR5′MP, dihydrozeatin riboside 5′-monophosphate; *iP*, N^6-(Δ^2isopentenyl)adenine; *iPR*, N^6-(Δ^2isopentenyl) adenosine; *iP9G*, N^6-(Δ^2isopentenyl)adenine 9-glucoside; *iPRMP*, N^6-(Δ^2isopentenyl)adenosine 5′-monophosphate. *B,* content of various forms of *cis*-zeatin. The legend is the same as in *A. C,* content of various forms of dihydrozeatin. The legend is the same as in *A. D,* content of various forms of N^6-(Δ^2isopentenyl)adenine. The legend is the same as in *A*.

type plants (data not shown). This result indicates that the CBF/DREB pathway is not coupled to cold-responsive TCS.

Cold-responsive Expression of A-type ARRs in Transgenic Arabidopsis Deficient in Cytokinins—We next tested if cold induces the expression of the *AHK* genes encoding cytokinin receptors, thereby resulting in up-regulation of cold-responsive *ARR*s. Our quantitative time course analysis using real time RT-PCR showed that cold did not induce *AHK2*, *AHK3*, and *AHK4* expression in a statistically significant manner within 24 h (supplemental Fig. 5). Previous RNA-gel blot analyses had also shown that cold did not significantly increase the expression of *AHK2*, *AHK3*, and *AHK4* in general, although some expression of these *AHK* genes at 2 h of cold treatment had been detected, whereas dehydration strongly induced the expression of *AHK2* and *AHK3* (14). As we have reproducibly observed significant cold-responsive *ARR* expression within 30 min and strong transient gene expression (Fig. 2), both results demonstrated that cold-responsive *ARR* expression is not primarily due to the increase in gene expression of these *AHK*s.

We then investigated whether cold can activate the enzymes involved in the biosynthesis of cytokinins, thereby resulting in increased cytokinin levels that subsequently activate the AHK receptors for A-type *ARR* expression as an early response. To test this possibility, we first attempted to determine whether decreasing levels of cytokinins can affect the cold-induced expression of A-type *ARR* genes via the use of cytokinin-deficient transgenic *Arabidopsis* plants. We utilized *35S:AtCKX2-2* plants that overexpress cytokinin oxidase and that contain less than 20% of total zeatin content and less than 40% of total cytokinin content compared with that of the wild-type plants (38). *AtCKX2-2* is the most cytokinin-deficient *35S:AtCKX* transgenic *Arabidopsis* among the *35S:AtCKX* plants generated (38). We treated these cytokinin-deficient transgenic plants with cold for 0, 1, 2 or 4 h and assessed the expression of *ARR5*, *ARR6*, *ARR7*, and *CBF1* by quantitative real time RT-PCR, as compared with the wild-type plants. Our results demonstrated that the expressions of those *ARR*s and *CBF1* were not influenced significantly by cytokinin deficiencies, in general (Fig. 4). Although we noted a statistically significant difference in cold-responsive *ARR5* expression between the wild-type and cytokinin-deficient transgenics, this difference was marginal and was observed only in cases of cold treatment applied for 4 h.

Cold-responsive Expression of A-type ARRs in Transgenic Arabidopsis Overproducing Cytokinins in a Dexamethasone-dependent Fashion—We next attempted to determine whether increased cytokinin levels might exert an effect on the cold-responsive expression of A-type *ARR*s. To prevent severe effects of constitutively overproduced cytokinins on plant growth and development, we utilized a transgenic *Arabidopsis* (pV-*ipt*/LhGR-N(ipt5-2)), which overexpresses isopentenyltransferase, a critical enzyme for cytokinin biosynthesis, and thus cytokinins in a DEX-inducible manner (39). The pV-*ipt*/LhGR-N transgenic plants contain an activator construct that expresses LhGR and a construct harboring isopentenyltransferase and *GUS* under the control of six copies of the ideal *lac* operator (39). DEX treatment results in the activation of both *IPT* and *GUS* genes (supplemental Fig. 6). Whereas DEX treatment applied for 14 days after germination induced no phenotypic change in the wild-type plants as well as the GR-overexpressing transgenic plants (LhGR-N), the same treatment induced severe growth inhibition in the pV-*ipt*/LhGR-N transgenic plants. However, when we applied DEX to 10-day-old pV-*ipt*/LhGR-N plants as well as two control plants, Col-0 and

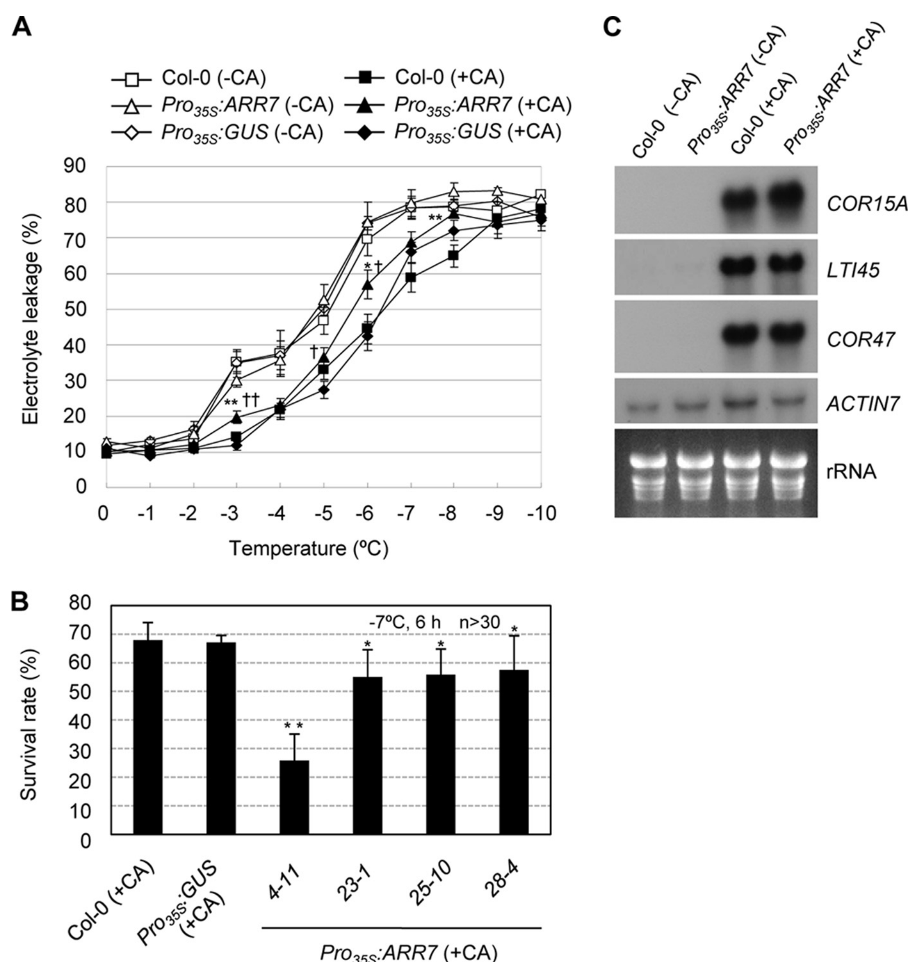

FIGURE 7. **Freezing tolerance assays and RNA-gel blot analysis of *Pro$_{35S}$:ARR7*, *Pro$_{35S}$:GUS*, and the wild-type plants with or without cold acclimation.** *A,* electrolyte leakage of *Pro$_{35S}$:ARR7* and the wild-type plants with or without cold acclimation. Electrolyte leakage assays were conducted using the leaves from 2-week-old wild-type (Col-0) and *Pro$_{35S}$:ARR7* (*line 4-11*) (29) plants treated with or without cold acclimation (*CA*). For cold acclimation treatment, the plants were incubated for 3 days at 1 °C under white fluorescent light. The means ± S.E. from 10 experiments were plotted. Statistically significant changes compared with those of Col-0 plants (or *Pro$_{35S}$:GUS* plants) are indicated by * (or †) when $p < 0.05$ or by ** (or ††) when $p < 0.01$ (Student's *t* test), respectively. *B,* freezing tolerance assays of various lines of *Pro$_{35S}$:ARR7* transgenic *Arabidopsis*, *Pro$_{35S}$:GUS*, and the wild-type plants after cold acclimation. Ten-day-old plants were cold-acclimated at 1 °C for 3 days, treated with −7 °C for 6 h, and photographed after 2 days of incubation at 23 °C for recovery. The percentage of the plants that survived was calculated. The means ± S.E. from four independent experiments were plotted. $n \geq 30$. # indicates line number of *Pro$_{35S}$:ARR7* (29). Statistically significant changes compared with the Col-0 plants as well as *Pro$_{35S}$:GUS* plants are indicated by * when $p < 0.05$ or by ** when $p < 0.01$ (Student's *t* test), respectively. *C,* RNA-gel blot analysis of *Pro$_{35S}$:ARR7* and the wild-type plants. Total RNA was isolated from plants treated with or without cold acclimation and was subjected to RNA-gel blot analysis using the indicated probes. *Line 4-11* of *Pro$_{35S}$:ARR7* (29) was utilized.

LhGR-N, for up to an additional 6 days, we could not note a significant inhibition in plant growth (supplemental Fig. 7).

Ten-day-old plants were therefore incubated with DEX for an additional 2, 4, or 6 days, followed by cold treatment at 1 °C for 0, 1, 2 or 4 h. Total RNAs extracted from the treated plants were subjected to comparative RT-PCR analysis. *ARR5* was selected for expression analysis as a representative *ARR* because of highly inducible gene expression to both cytokinins and cold. As shown in Fig. 5*A*, the preincubation of the pV-*ipt*/LhGR-N plants with DEX enhanced *ARR5* expression levels as compared with what was observed in the absence of DEX, whereas two control plants were unresponsive to DEX, thereby indicating the ability of cytokinins to activate *ARR5* expression in the pV-*ipt*/LhGR-N plants. The application of cold treatment to all three of these plants resulted in increased *ARR5* expression. We next conducted real time RT-PCR analysis to quantify the response of *ARR5*, *ARR6*, and *ARR7* to cold with plants incubated for 4 days with DEX (Fig. 5*B*). DEX clearly induced *ARR5*, *ARR6*, and *ARR7* expression (Student's *t* test, $p < 0.001$). Similar patterns were noted on comparative RT-PCR (data not shown). In the case of *ARR5*, DEX treatment exerted additive effects on the expression of cold-responsive genes after 1 h of cold treatment, but the expression levels reached a plateau after 2 h of treatment. *ARR6* showed maximum expression levels after 1 h of cold treatment. *ARR7* exhibited additive induction kinetics with both cold and DEX treatment for up to 2 h of cold treatment. These results showed that increasing levels of cytokinins exert additive effects with cold on *ARR5*, *ARR6*, and *ARR7* expression at early time points of the treatment, but the maximum induction levels reached by the treatment of both cold and DEX were identical to those of cold treatment alone.

Cytokinin Analysis of Arabidopsis Subjected to Cold Treatment Showed Insignificant Changes in Cytokinin Levels during Peak Expression Time of Cold-responsive ARRs—To determine whether cold could induce an increase in cytokinin levels, thereby mediating A-type *ARR* expression, we directly measured the amount of a variety of different forms of cytokinins in 10-day-old wild-type plants that had been subjected to 0, 0.5, 1, 2, or 4 h of cold treatment at 1 °C, at which the maximal peak expression of cold-inducible A-type *ARR* genes was observed, as shown in Fig. 2. We determined that none of the cytokinins evidenced a meaningful increase, which can account for the cold-inducible expression of *ARR* genes (Fig. 6 and supplemental Table 2). This result shows that an increase in cytokinin levels is not the cause of the expression of A-type *ARR* genes as an early response.

ARR7 Overexpression Results in Hypersensitive Response in Transgenic Arabidopsis to Freezing Temperatures after Cold Acclimation—To determine whether cold-inducible A-type *ARR*s might perform a function in stress tolerance response, we conducted electrolyte leakage assays for transgenic *Arabidopsis* overexpressing *ARR7* (*Pro$_{35S}$:ARR7*) (29). Under normal conditions, *Pro$_{35S}$:ARR7* (line 4-11) and both control plants, *Pro$_{35S}$*:

GUS and the wild type (Col-0), showed similar ion leakage patterns (Fig. 7*A*). After cold acclimation, Pro_{35S}:*ARR7* exhibited hypersensitive ion leakage patterns to freezing temperatures compared with two control plants, the wild type at −3, −5, −6, and −8 °C and Pro_{35S}:*GUS* at −3, −5, and −7 °C, to some extent, indicating the negative regulator activity of ARR7 against freezing stress. *In planta* freezing tests using additional three Pro_{35S}:*ARR7* lines yielded similar results, albeit with weaker effects than were observed in line 4-11 (Fig. 7*B*). The weaker effects might be attributable to the lower ARR7 activity of these transgenic lines compared with line 4-11 with regard to the repression of its target genes, as reported previously (29). Cold treatment for 2 days efficiently induced a variety of cold-regulated genes in the CBF/DREB pathway of both Pro_{35S}:*ARR7* and control plants (Fig. 7*C*), thereby suggesting that ARR7 may function as a negative regulator of cold signaling, independent of CBF/DREB cold signaling.

Loss-of-Function Mutations in ARR5, ARR6, or ARR7 Cause Increased Freezing Tolerance—The hypersensitive freezing tolerance of the *ARR7*-overexpressing lines compelled us to assess whether the loss-of-function mutations in cold-inducible *ARR* genes could induce enhanced freezing tolerance. Whereas *arr5* and *arr6* are null mutants (28), *arr7* null mutants are not available. We found transposon T-DNA insertion lines of *ARR7* where the mRNA levels were reduced by up to more than 20-fold compared with that of the wild type (supplemental Fig. 1). We determined the survival rates of *arr5*, *arr6*, or *arr7* after 5 h of freezing at −5 °C, and we noted that *arr5* and *arr7* evidenced statistically significantly increased freezing tolerance, occurring to some extent even prior to cold acclimation (Fig. 8*A*). When these mutants were cold-acclimated, significantly increased freezing tolerance in *arr5*, *arr6*, and *arr7* were noted after 7 h of freezing at −8 °C. In particular, *arr6* and *arr7* exhibited greatly enhanced freezing tolerance at survival rates in excess of 50% as compared with the wild-type plants at 10% (Fig. 8*B*). These results indicate that cold-inducible *ARR* genes may function as a negative regulator of cold signaling. In addition, we measured the *AHK2* and *AHK3* transcript levels by real time RT-PCR in *arr5*, *arr6*, *arr7*, and Pro_{35S}:*ARR7* as compared with the wild type, but we could not observe detectable differences in the transcript levels between these mutants and the wild-type plants (supplemental Fig. 8), suggesting that regulation of *AHK2* and *AHK3* gene expression is not related to alteration in cold tolerance in these mutants.

Freezing Tolerance of ahk Mutants—We then analyzed the freezing tolerance of *ahk* mutants to determine whether the AHK-cytokinin receptors mediating A-type *ARR* expression perform a function in cold response. As the single *ahk* mutants did not evidence any significant phenotypes, we first evaluated the freezing tolerance of the *ahk* double mutants, *ahk2-2 ahk3-3* and *ahk3-3 cre1-12*, in the Col-0 ecotype backgrounds (9). We tested a variety of freezing temperatures and incubation times. As shown in Fig. 9, *A* and *B*, *ahk2-2 ahk3-3* and *ahk3-3 cre1-12* exhibited significantly higher survival rates against freezing temperatures compared with the wild-type control plants, and *ahk2-2 ahk3-3* displayed more profound freezing tolerance than was seen with the *ahk3-3 cre1-12* line. A different allele, *ahk2-1 ahk3-1* mutant, also showed similarly

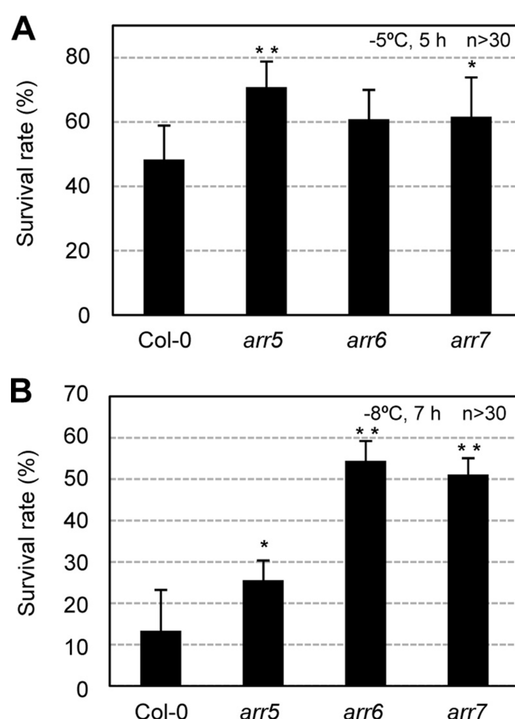

FIGURE 8. **Freezing tolerance assays of A-type *arr* mutants compared with the wild-type plants with or without cold acclimation.** *A,* freezing tolerance assays of the A-type *arr* mutants prior to cold acclimation. Freezing assays were performed on the wild-type (Col-0), *arr5*, *arr6*, and *arr7* mutants, as described in the Fig. 7*B* legend except for 5 h of freezing treatment at −5 °C. Statistically significant changes compared with the Col-0 plants are indicated by * when $p < 0.05$ or by ** when $p < 0.01$ (Student's *t* test), respectively. *B,* freezing tolerance assays of the A-type *arr* mutants after cold acclimation. Freezing assays were performed on the wild-type (Col-0), *arr5*, *arr6*, and *arr7* mutants after cold acclimation, as described in the Fig. 7*B* legend except for freezing treatment at −8 °C for 7 h. Statistically significant changes compared with the Col-0 plants are indicated by * when $p < 0.05$ or by ** when $p < 0.01$ (Student's *t* test), respectively.

enhanced freezing tolerance compared with the wild-type plants (data not shown). However, the *ahk2-2 cre1-12* plants showed insignificant changes in freezing tolerance compared with the wild type (data not shown). These results show that the AHK-cytokinin receptors may function partially redundantly as a negative regulator of the cold stress adaptation response.

To ascertain whether the constitutive freezing tolerance of *ahk* mutants might be attributable to acquired cold acclimation, we investigated the ability of *ahk* mutants to acclimate to cold as compared with the wild-type plants. We incubated the plants for 3 days at 1 °C for cold acclimation and then treated them for 6 h with freezing temperatures of −7 °C. As shown in Fig. 9, *C* and *D*, the *ahk2-2 ahk3-3* and *ahk3-3 cre1-12* double mutants exhibited significantly higher survival rates against freezing temperatures compared with the wild-type control plants after cold acclimation, and the *ahk2-2 ahk3-3* lines displayed higher freezing tolerance than that of *ahk3-3 cre1-12*. The *ahk2-1 ahk3-1* double mutants also exhibited similarly enhanced freezing tolerance compared with the wild-type plants (data not shown). We also determined expression levels of *COR15A* and *COR47*, the marker genes of CBF/DREB pathway during cold acclimation, and found that the levels of these transcripts before and after cold acclimation were not changed by *ahk2 ahk3* and *ahk3 cre1* double mutations (Fig. 10). Similar

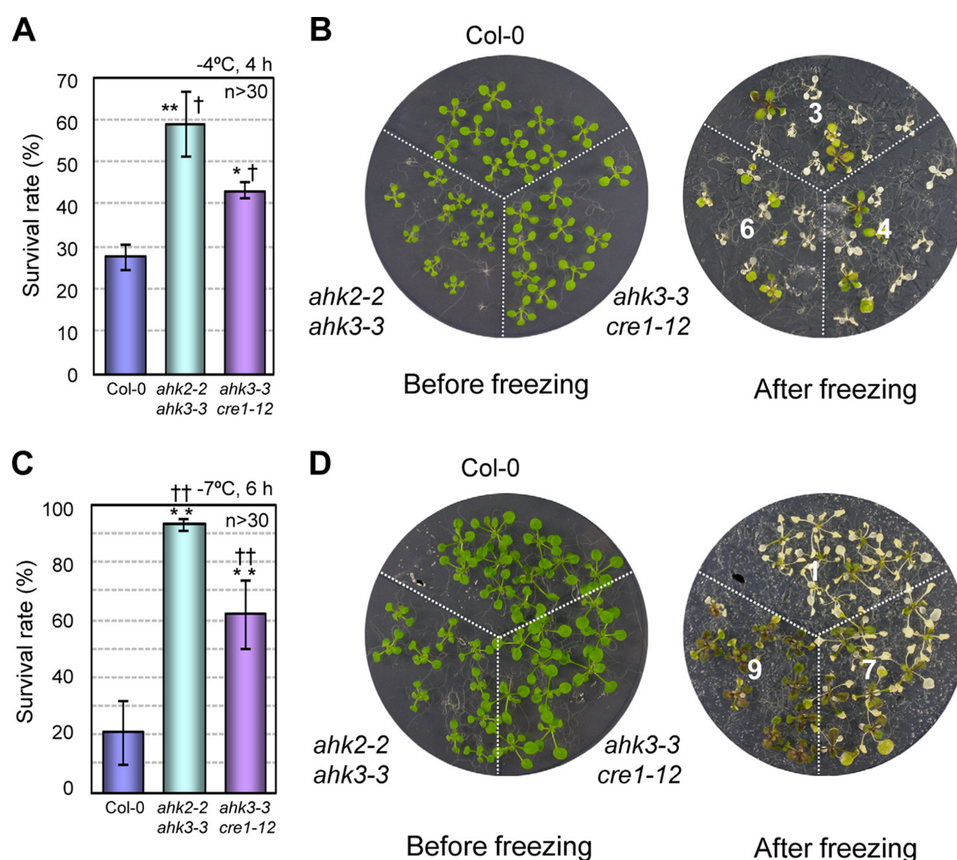

FIGURE 9. **Freezing tolerance assays of *ahk* double mutants compared with the wild-type plants with or without cold acclimation.** *A, ahk2-2 ahk3-3* and *ahk3-3 cre1-12* mutants survived after 4 h of freezing at −4 °C. Fourteen-day-old light-grown plants were treated for 4 h at −4 °C and photographed after 2 days of incubation at 23 °C for recovery. The percentage of the plants that survived was calculated. Experiments were conducted in triplicate, and the means ± S.E. were plotted. $n \geq 30$. All mutants are derived from the Col-0 ecotype. Statistically significant changes compared with Col-0 plants were indicated by * when $p < 0.05$ or by ** when $p < 0.01$ (Student's *t* test), respectively. † denotes statistically significant changes with $p < 0.05$ among the *ahk* double mutants indicated. *B,* example plates showing plants subjected to freezing tolerance assays (*A*). One plate (inner diameter 150 × 20 mm) contains 10 plants per each control or mutant. *C,* treatment of the *ahk2-2 ahk3-3* and *ahk3-3 cre1-12* mutants cold-acclimated with freezing at −7 °C for 6 h. Fourteen-day-old light-grown seedlings were cold-acclimated for 3 days, treated at −7 °C for 6 h, and the plants that survived were counted after 2 days of incubation at 23 °C for recovery. The percentage of the plants that survived was calculated. Experiments were conducted in triplicate, and the means ± S.E. were plotted. $n \geq 30$. Statistically significant changes with $p < 0.01$ (Student's *t* test) compared with the Col-0 plants are indicated by **. †† denotes statistically significant changes with $p < 0.01$ among *ahk* double mutants indicated. *D,* example plates showing plants subjected to freezing tolerance assays (*C*). One plate (inner diameter 150 × 20 mm) contains 10 plants per control or per mutant.

patterns were noted on comparative RT-PCR (data not shown). These results suggest that enhanced freezing tolerance in the *ahk2 ahk3* or *ahk3 cre1* double mutants is not directly linked to the CBF/DREB pathway.

Preincubation of ahk Single Mutants with Cytokinin Caused Enhanced Freezing Tolerance Compared with Untreated Mutants as Well as the Wild Type Treated with Cytokinin—To further demonstrate that the AHK-cytokinin receptors act as a negative regulator of cold stress adaptation response, we tested the effects of cytokinin preincubation on freezing tolerance of *ahk* single mutants that did not show significant freezing tolerance compared with the wild type. We incubated 3-day-old *ahk* single mutants, *ahk2-1*, *ahk3-1*, and *cre1-12*, and wild-type plants in the presence of the cytokinin, kinetin, for an additional 24 h or 7 days and then conducted freezing tolerance assays. When incubated for 24 h, *ahk* single mutants and the wild-type plants exhibited enhanced freezing tolerance to some extent upon preincubation with cytokinin (Fig. 11, *A* and *B*). However, a longer treatment of cytokinins, for 7 days compared with 24 h, induced a profound enhancement of freezing tolerance in the *ahk* single mutants, particularly, *ahk2* and *ahk3*, as compared with the wild-type plants (Fig. 11, *C* and *D*). These results bolster the notion that the AHK-cytokinin receptors may function as a negative regulator of the cold stress adaptation response and that cytokinins might be involved in adaptation response to freezing temperatures.

Pro_{35S}:ARR7 Exhibited Enhanced Freezing Tolerance Compared with arr7 Mutants upon Cytokinin Preincubation—We next tested whether preincubation with cytokinin impacts the responsiveness of the *arr5*, *arr6*, and *arr7* mutants and *Pro_{35S}:ARR7* lines to cold, as compared with the wild type. Preincubation of *arr* mutants with cytokinin for 7 days resulted in increased freezing tolerance as much as that of the wild type (Fig. 12, *A* and *B*). Interestingly, *Pro_{35S}:ARR7* plants exhibited stronger freezing tolerance compared with the *arr7* mutants and two other control plants (Col-0 and *Pro_{35S}:GUS*) upon cytokinin preincubation (Fig. 12, *C* and *D*). As overexpression of *ARR7* in *Arabidopsis* reduces response to cytokinin (29), this result might indicate that proper levels of cytokinin signaling might be important for cold tolerance.

Response of Root Growth and Germination of arr5, arr6, arr7, Pro_{35S}:ARR7, and ahk2 ahk3 to ABA—It has been reported previously that ABA-regulated genes were up-regulated in the *ahk2-2 ahk3-3* mutants (14). The activation of ABA-regulated genes and observed enhancements of freezing tolerance by *ahk* mutations might be due to increased sensitivity in the ABA response. To test this possibility, we measured root growth in the presence of exogenous ABA compared with auxin (Fig. 13). The *ahk2-2 ahk3-3* mutants were significantly sensitive to ABA compared with the wild type at the ABA concentrations tested (Student's *t* test, $p < 0.01$) (Fig. 13*A*), whereas this mutant showed a normal response to auxin, similar to the wild type (Fig. 13*B*). We also measured root growth inhibition of *arr* mutants and *Pro_{35S}:ARR7* lines but observed statistically insignificant difference or very marginal difference compared with the wild type (Fig. 13*A*).

To further explore the ABA response of *arr* mutants, *Pro_{35S}:ARR7* lines, and *ahk2 ahk3* double mutants, we measured ger-

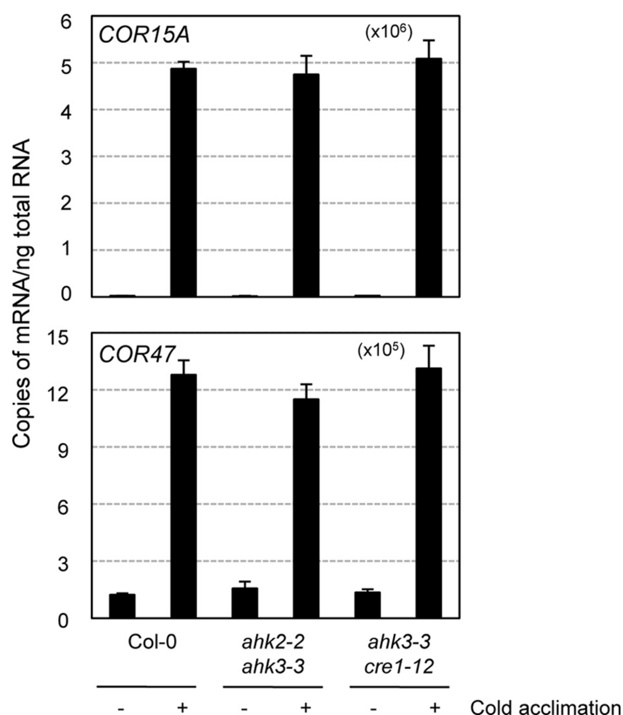

FIGURE 10. **Expression of *COR15A* and *COR47* in *ahk2 ahk3* mutants compared with the wild-type plants with or without cold acclimation.** Eleven-day-old light-grown seedlings were treated at 1 °C for 3 days for cold acclimation, and total RNAs isolated were subjected to real time RT-PCR. Copies of the transcripts from plants treated were plotted per ng of total RNA after normalization to *ACTIN7* RNA. The transcripts levels were determined as described in the Fig. 2 legend.

mination of these seeds with varying concentrations of ABA (Fig. 14). Consistent with root growth inhibition results, the *ahk2-2 ahk3-3* double mutants showed a sensitive response to ABA compared with the wild type. Strikingly, the *arr7* mutants exhibited the highest sensitive response to ABA among the seeds tested, whereas Pro_{35S}:*ARR7* lines showed the highest insensitive response to ABA. *arr6* also showed sensitive response to ABA compared with the wild type, whereas *arr5* displayed insensitive response to ABA to some extent. This result further demonstrates that there is a cross-talk between cytokinin and ABA signaling. Hypersensitive response of the *arr7* mutants and insensitive response of *ARR7* overexpression lines to ABA suggest that *ARR7* might play a role as a negative regulator in ABA signaling.

DISCUSSION

Cold-inducible A-type ARRs Function as a Negative Regulator of Cold Signaling—We noted the transient expression of a subset of A-type *ARR* genes, including *ARR5*, *ARR6*, *ARR7*, and *ARR15,* in response to cold temperatures. The *ARR7* promoter-*GUS* transgenic *Arabidopsis* expressed *GUS* in response to cold, as well as to cytokinins. These results are indicative of a potential function of A-type *ARR*s in cold signaling. We observed the hypersensitivity response of Pro_{35S}:*ARR7* plants with decreasing freezing temperatures to some extent upon cold acclimation, as compared with the control plants (Fig. 7). Various CBF/DREB pathway genes were shown to be equally inducible by cold in both the wild-type and Pro_{35S}:*ARR7* plants (Fig. 7). These results indicated that cold-inducible *ARR7* may play a role as a negative feedback regulator of cold stress signaling in the CBF/DREB-independent pathway. If a cold-inducible *ARR* gene does function as a negative regulator, then loss-of-function mutation in a cold-inducible *ARR* might induce an increase in freezing tolerance compared with the wild-type plants. Increased freezing tolerance has been noted in *arr5* and *arr7,* although the effects were weak, prior to cold acclimation and in *arr5*, *arr6*, and *arr7* after cold acclimation (Fig. 8). In particular, *arr6* and *arr7,* after cold acclimation, showed relatively stronger freezing tolerance than was observed with the wild-type and *arr5*. This result further supports the proposition that the cold-inducible *ARR* genes might function as a negative regulator of cold signaling.

AHK-Cytokinin Receptors Function as a Negative Regulator of Cold Signaling—The *ahk2 ahk3* and *ahk3 cre1* mutants exhibited tolerance to freezing temperatures compared with the wild-type plants prior to or after cold acclimation (Fig. 9). This result indicates that the AHK-cytokinin receptors might function as a negative regulator of the cold stress adaptation response, which is consistent with the negative regulator function of the cold-inducible A-type *ARR*s. The *ahk2 ahk3* mutants were shown to be more tolerant to freezing temperatures than the *ahk3 cre1* mutants. However, the difference between the freezing tolerance of the *ahk2 cre1* mutants and that of the wild type was not significant (data not shown). The reason that the freezing tolerance of the *ahk2 ahk3* mutants was higher than that of the *ahk3 cre1* or *ahk2 cre1* plants remains to be elucidated, but it may be associated with the prominent functions of *AHK2* and *AHK3* and, more critically, *AHK3* in the leaf and *AHK4* in the roots (9–11). Whereas the *ahk3 ahk4* or *ahk2 ahk4* double mutants and *ahk* single mutants grew normally, the *ahk2 ahk3* mutants exhibited symptoms of semi-dwarfism, including short petioles and smaller leaf blades (9, 10). The *ahk4* single mutants exhibited significant resistance to cytokinin in the root elongation assays compared with the wild type, but the *ahk2* or *ahk3* or *ahk2 ahk3* mutants did not. Consistent with this, *AHK4* was expressed predominantly in the roots (9, 10). These results imply that *AHK2* and *AHK3* may be critical to leaf development and function, whereas *AHK4* may function principally in the roots. The leaf may be the primary site used by the plants to cope with cold stress. Thus, the degree of freezing tolerance can be predicted in the following order on the basis of this rationale and the hypothesis that AHK3 might be more important than AHK2 in the cold-stress adaptation response: *ahk2 ahk3* > *ahk3 ahk4* > *ahk2 ahk4*. The inhibition of leaf development conferred by *ahk2 ahk3* mutations may constitute a more stringent barrier against environmental cold stress over the ground. However, the *ahk3 ahk4* double mutants grew normally (9, 10), even though this mutant exhibited enhanced freezing tolerance. The *fatb* mutants that exhibited significant dwarfism and small leaves (51) also showed a more sensitive phenotype to freezing stress than was observed in the wild-type plants (data not shown). Thus, the freezing tolerance exhibited by the *ahk2 ahk3* double mutants appears not to be due solely to the reduction in leaf size.

To assess the connection between freezing tolerance and negative regulation of cytokinin signal transduction, we have investigated the effects of cytokinins on the freezing tolerance

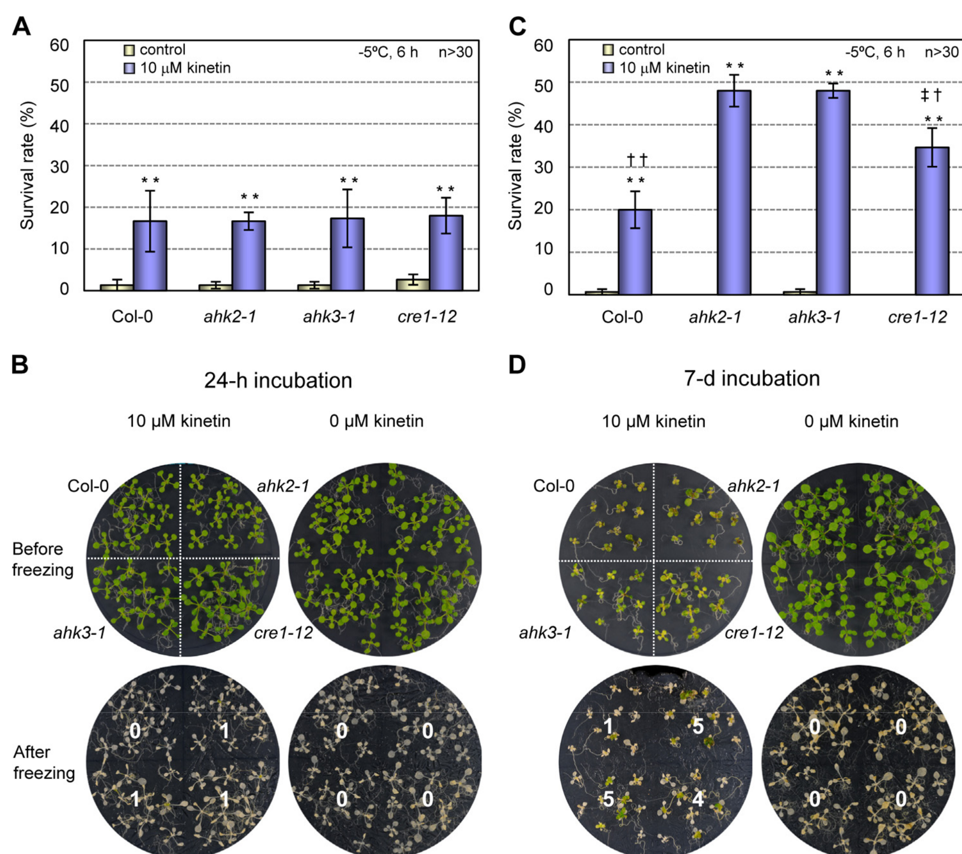

FIGURE 11. **Effect of cytokinin preincubation on freezing tolerance of *ahk2*, *ahk3*, and *cre1* single mutants compared with the wild-type plants.** *A, ahk* single mutants preincubated with or without cytokinin for 24 h. The *ahk2-1*, *ahk3-1*, *cre1-12* mutants and the wild-type plants (Col-0) were grown for 9 days in the light, transferred to MS plate or MS plate containing 10 μM kinetin, and grown for an additional 24 h. These 10-day-old plants preincubated with or without cytokinin were subjected to freezing treatment at −5 °C for 6 h. The plants that survived after incubation at 23 °C for 2 days for recovery were counted. Experiments were conducted in triplicate, and the means ± S.E. were plotted. $n \geq 30$. Statistically significant change with $p < 0.01$ (Student's *t* test) compared with the samples without cytokinin treatment is indicated by **. †† denotes statistically significant changes with $p < 0.01$ among *ahk* single mutants indicated. *B,* example plates showing the *ahk2-1*, *ahk3-1*, *cre1-12* mutants, and the wild-type plants preincubated with or without cytokinin for 24 h, subjected to freezing treatment (*A*). *C, ahk* single mutants preincubated with or without cytokinin for 7 days. Plants were treated and analyzed as described in *A* except for 3 days growth followed by 7 days of incubation with cytokinin. *D,* example plates showing the *ahk2-1*, *ahk3-1*, *cre1-12* mutants and the wild type plants preincubated with or without cytokinin for 7 days and then subjected to freezing treatment. Plant growth and cytokinin preincubation were conducted as described in *C*.

of *ahk* single mutants of the undetectable freezing tolerance phenotype, via the preincubation of the mutants with cytokinin prior to freezing. We determined that preincubation with cytokinin resulted in detectable levels of enhanced freezing tolerance in the *ahk2*, *ahk3*, or *cre1* mutants as well as the wild type even at 24 h. However, longer treatment of cytokinin such as for 7 days caused significantly enhanced freezing tolerance in *ahk* single mutants as well as the wild type compared with untreated plants and also induced a greater increase in freezing tolerance in the *ahk* single mutants than in the wild-type plants (Fig. 11). This result shows that exogenous cytokinins and cytokinin signal transduction both play a role in the acquisition of freezing tolerance in *ahk* mutants. Whereas the relevant molecular mechanisms remain to be clearly elucidated, exogenous cytokinins applied to the *ahk* single mutants might strengthen the negative feedback inhibition of cytokinin signal transduction, thereby resulting in enhanced freezing tolerance. This situation may, in part, resemble higher order mutations in *AHKs* that induced significantly increased freezing tolerance, even in the absence of exogenous cytokinin treatment. Similar observations were also previously noted in *cre1* mutants, which exhibited a salt-tolerant phenotype in the presence of exogenous cytokinins (14).

We further investigated the impact of cytokinin preincubation on the response of *arr* mutants to cold and observed similarly increased freezing tolerance in *arr5*, *arr6*, and *arr7* as well as in the wild type (Fig. 12). However, significantly enhanced freezing tolerance in *ARR7* overexpression lines was found as compared with the *arr7* mutants upon cytokinin preincubation. *ARR7* overexpression caused insensitive cytokinin response similar to other A-type *ARR* genes (29). *ARR7* overexpression in Pro_{35S}:*ARR7* might have then reduced the effects of cytokinin exogenously applied by cytokinin preincubation. This inhibition effect might have been beneficial for Pro_{35S}:*ARR7* plants compared with the plants such as the *arr7* mutants and the wild type exogenously added with cytokinin showing slight growth inhibition. At any rate, it is clear that certain levels of cytokinin and thus cytokinin signal transduction render the plants to be tolerant to cold.

Acquired cold acclimation might be one mechanism to explain the enhancement of freezing tolerance in *ahk2 ahk3* or *ahk3 ahk4* double mutants. However, our results showed that these mutants have the ability to acclimate to cold like wild-type plants and that the marker genes for cold acclimation such as *COR15A* and *COR47* in CBF/DREB pathway are equally inducible in these mutants and the wild-type plants (Fig. 10). It is therefore unlikely that cold acclimation process is primarily involved in cold-stress response mediated by the AHK-cytokinin receptors. Another explanation is that endogenously increased levels of cytokinins in the *ahk* mutants might be responsible for the differing levels of freezing tolerance observed among *ahk* mutants. However, no linear correlation between freezing tolerance and endogenous cytokinin levels was detected in the *ahk* mutants (11). For example, *ahk3*, *ahk2 ahk3*, and *ahk3 cre1* harbor similar cytokinin levels, although the *ahk* single mutants displayed wild-type like freezing tolerance and *ahk2 ahk3* exhibited stronger freezing tolerance than *ahk3 cre1*. Thus, endogenously increased cytokinin levels due to single or double mutations in *AHK*s might not be

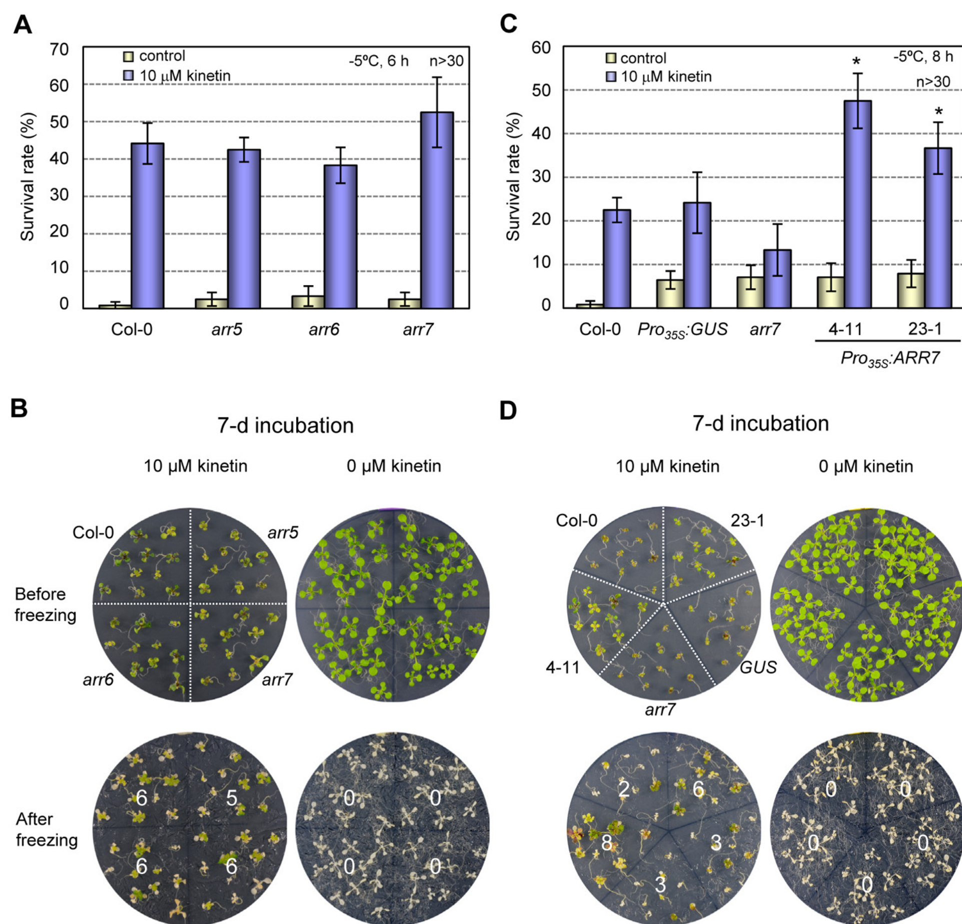

FIGURE 12. **Effect of cytokinin preincubation on freezing tolerance of *arr5*, *arr6*, *arr7* single mutants and *Pro35S:ARR7* lines compared with the wild-type plants.** *A*, *arr5*, *arr6*, and *arr7* mutants preincubated with or without cytokinin for 7 days. Experiments were conducted and analyzed as described in Fig. 11, *C* and *D*. *B,* example plates showing the *arr5*, *arr6*, *arr7* mutants, and the wild-type (Col-0) plants preincubated with or without cytokinin for 7 days, subjected to freezing treatment (*A*). *C*, *Pro35S:ARR7* lines compared with the *arr7* mutants and Col-0 and *Pro35S:GUS* control plants preincubated with or without cytokinin for 7 days. Experiments were conducted and analyzed as described in *A* except that the plants were treated at −5 °C for 8 h for freezing. Numbers *4-11* and *23-1* indicate the line number of *Pro35S:ARR7* transgenic plants. * denotes statistically significant change with $p < 0.05$ compared with *arr7* and control plants pretreated with cytokinin. *D,* example plates showing *Pro35S:ARR7* lines, the *arr7* mutants, Col-0, and *Pro35S:GUS* control plants preincubated with or without cytokinin for 7 days and then subjected to freezing treatment (*C*).

responsible for enhancing freezing tolerance in these *ahk* mutants, as compared with exogenously added cytokinins.

Alteration of expression of biotic and abiotic stress-related and some auxin-induced genes by overexpression of $ARR2^{D80E}$ compared with *ARR2* overexpressor or by *arr2* mutation has been reported (52). Activation of ABA up-regulated genes has been observed in the *ahk2 ahk3* double mutants (14). Thus enhanced ABA response might be one explanation for increased freezing tolerance in the *ahk2 ahk3* mutants. This prediction is consistent with hypersensitive ABA response of the *ahk2 ahk3* mutants in both root growth inhibition and germination inhibition (Fig. 13*A* and Fig. 14). Germination assays have previously shown that the cytokinin receptor single mutants are hypersensitive to ABA (14). It has been proposed that the increased cytokinin content might antagonistically regulate ABA synthesis, inhibit ABA action in germinating seeds through cytokinin receptors, and consequently alleviate the inhibition effect of ABA on seed germination (14). These results indicate that the AHK-cytokinin receptors might be involved in regulating interaction between cytokinin and ABA signaling. Moreover, a parallel phenotype of *arr7* and *Pro35S:ARR7* in germination assays (Fig. 14) has indicated that *ARR7* might play a role as a negative regulator in ABA signaling. These results suggest that cross-talk might exist between cytokinin and ABA signaling in an antagonistic fashion for regulating cold stress response.

AHK-Cytokinin Receptors Mediate Cold Signals for Inducing A-type ARR Expression—Severe inhibition of cold-responsive A-type *ARR* expression in the *ahk2 ahk3* mutants compared with the wild-type plants (Fig. 3) may be associated with the *in vivo* functioning of the AHK-cytokinin receptors in plants. Severely reduced expression of A-type *ARR*s in response to cold was noted in the *ahk2 ahk3* mutants, but their normal cold response in the *ahk* single mutants suggests that *AHK2* and *AHK3* might have redundant functions and constitute the principal mediators of cold-responsive A-type *ARR* expression. The normal cold-responsive expression of *ARR* genes was noted in the *ahk3 ahk4* and *ahk2 ahk4* mutants (Fig. 3*B*). These results suggest that AHK2 may perform redundant functions with AHK3 in cold perception as well as in cytokinin reception, whereas AHK4 may perform a specific function in cytokinin reception. Thus, loss-of-function of *AHK2* or *AHK3* may not be replaced with *AHK4* in cold perception, thereby resulting in severe inhibition of cold-responsive *ARR* expression than that of *ahk3 ahk4* or *ahk2 ahk4,* which displays near wild-type levels of cold-responsive *ARR* expression. Although *ahk3 ahk4* showed the normal cold-responsive expression of *ARR* genes (Fig. 3*B*), *ahk3 ahk4* exhibited detectable freezing tolerance compared with the wild type, albeit weaker than *ahk2 ahk3* (Fig. 9). These results indicate that in addition to the negative regulatory function mediated by the cold-inducible A-type *ARR*s, other signaling networks might be superimposed onto the cold response regulated downstream of AHKs.

The analysis of transgenic plants overproducing cytokinins in a DEX-inducible manner (Fig. 5) demonstrated that increased cytokinin levels exerted additive effects on the expression of *ARR5*, *ARR6*, and *ARR7* in response to cold. The maximum induction levels achieved by treatments with both cold and DEX were identical to those treated solely with cold. These results show that cold and cytokinins might share the

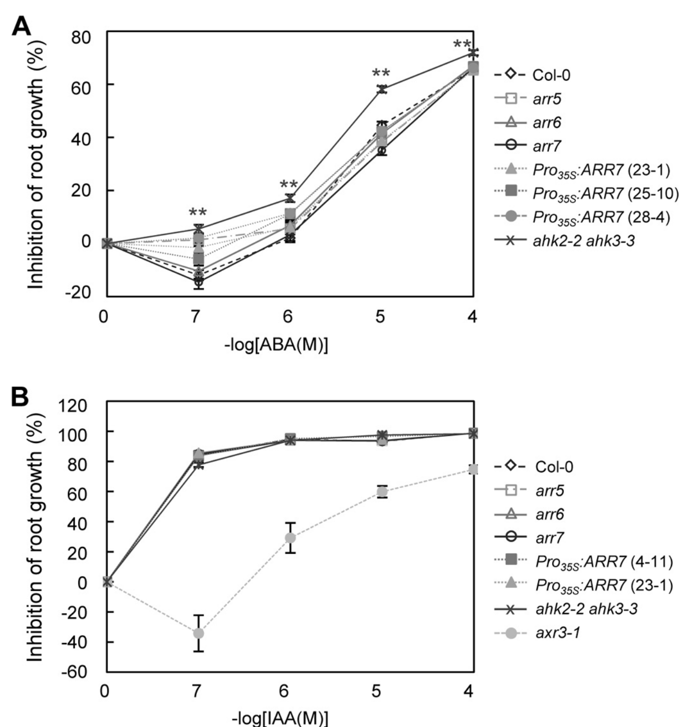

FIGURE 13. **Root growth of *arr5, arr6, arr7* mutants, *Pro*$_{35S}$*:ARR7* lines, and *ahk2 ahk3* double mutants compared with the wild-type plants in the presence of exogenous ABA or auxin.** *A,* analysis of primary root growth of *arr5*, *arr6*, *arr7*, *Pro*$_{35S}$*:ARR7*, and *ahk2 ahk3* in the presence of exogenous ABA. The seedlings, the wild type (Col-0), *arr5*, *arr6*, *arr7*, three different *Pro*$_{35S}$*:ARR7* transgenic lines, and *ahk2-2 ahk3-3* double mutants, were grown on MS agar plates for 4 days, transferred to the medium containing increasing concentrations of ABA, and grown vertically for additional 3 days. The root lengths were measured, and the mean values were plotted. $n \geq 26$. *Bars* indicate S.E. Statistically significant change with $p < 0.01$ (Student's *t* test) compared with the wild type are indicated by **. *B, arr5*, *arr6*, *arr7*, *Pro*$_{35S}$*:ARR7*, and *ahk2 ahk3* in the presence of auxin. Measurements of primary root-growth in the presence of auxin were performed as described in *A*. $n \geq 26$.

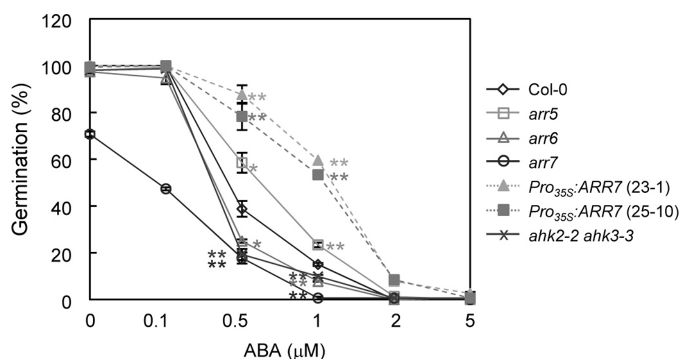

FIGURE 14. **Germination of *arr5, arr6, arr7* mutants, *Pro*$_{35S}$*:ARR7* lines, *ahk2 ahk3* double mutants, and the wild-type plants in the presence of exogenous ABA.** Seeds of the wild type (Col-0), *arr5*, *arr6*, *arr7*, two different *Pro*$_{35S}$*:ARR7* transgenic lines, and *ahk2-2 ahk3-3* were plated on MS medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4 °C in darkness, and incubated for 2 days at 21 °C with a 16-h light photoperiod. The number of germinated seeds was expressed as the percentage of the total number of seeds plated (63). Statistically significant change with $p <$ 0.05 (Student's *t* test) or $p < 0.01$ compared with the wild type is indicated by * or **.

same AHK-cytokinin receptors for the regulation of *ARR5*, *ARR6*, and *ARR7* expression. Additional AHK proteins, such as AHK1 and AHK5, may be involved in mediating cold to express A-type *ARR*s. Nevertheless, it is clear that AHK2 and AHK3 are the primary receptors for mediating the cold-inducible expression of A-type *ARR* genes.

Cold Perception of the AHK Proteins as a Primary Response Does Not Involve Changes in Cytokinin Levels for the Expression of A-type ARRs—The mechanisms by which the AHK-cytokinin receptors mediate cold temperatures for *ARR* expression remain to be clearly elucidated. Lack of significant cold-responsive expression of *AHK2*, *AHK3*, and *AHK4* (supplemental Fig. 5) suggests that the AHK-cytokinin receptor proteins might be involved in mediating cold temperatures for the expression of cold-responsive A-type *ARR* genes. Cytokinins themselves may be involved in cold-inducible A-type *ARR* expression. However, on the basis of our observations described below, we think that increased cytokinin levels might not be involved in the cold perception process during early response of A-type *ARR* gene expression. *ARR5*, *ARR6*, *ARR7*, and *ARR15* have characteristics of primary response genes in response to cold. Unfortunately, the immediate response of A-type *ARR* genes to cold without protein synthesis cannot be verified experimentally, because of the significant up-regulation of *ARR7* resulting from treatment with cycloheximide (48). Enzymes relevant to cytokinin biosynthesis could be cold-activated and may be involved in the cold perception process. However, if cold induces A-type *ARR* expression via cytokinins, the same set of *ARR* inductions in response to cold and cytokinins would be anticipated, which was obviously not the case. Moreover, our results demonstrated that cytokinin-deficient transgenic *Arabidopsis* plants exhibit a cold-responsive expression of A-type *ARR* genes, similar to that observed with the wild-type plants (Fig. 4). Cytokinins and cold exerted additive effects on A-type *ARR* gene expression (Fig. 5). Finally, direct measurements of the amount of a variety of different forms of cytokinins demonstrated that cold did not cause a significant increase in cytokinin levels within the time period (~4 h) during which a peak expression of *ARR* genes to cold temperatures has been observed (Figs. 2 and 6), thereby demonstrating that an increase in cytokinin levels might not be involved in the process of the cold perception of the AHK proteins for A-type *ARR* expression as an early response.

A potential sensor for cold perception in cyanobacteria, *Synechosystis* sp. PCC6803, has been proposed previously (53). Cyanobacteria modulate the composition of membrane lipids in response to shifts in temperature from 34 to 22 °C to increase the fluidity of their membranes for adaptation to cold stress by enhancing the expression of three fatty-acid-desaturase genes (*des*), *desA*, *desB*, and *desD* (54). Reductions in membrane fluidity via the catalytic hydrogenation of fatty acids in the plasma membranes of *Synechosystis* resulted in the induction of *desA* gene transcription (55), thus suggesting that the increased expression of the desaturase genes in response to cold stress might be regulated by the degree of membrane rigidity. Murata and co-workers later identified histidine kinase (Hik), Hik33 as a sensor for cold perception (56). Interestingly, Hik33 has been shown to regulate the expression of osmotic stress-inducible genes and also to bind to certain chemicals, indicating that it may function as a multifunctional sensor for a variety of stresses. Several stud-

ies have previously shown that changes in membrane fluidity appear to be a potential mechanism for the sensing of cold temperatures in higher plants as well (57–60). As proposed for bacterial Hik33 (61), it can be speculated that the plant AHK proteins might utilize a similar mechanism for the detection of cold temperatures. However, further biochemical studies will be necessary to clearly elucidate the mechanisms by which the AHK proteins contribute to the process of cold temperature recognition in higher plants.

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