

ARF7/19 activate *CRF3* in response to cold via Aux/IAA degradation^{oo}

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CYTOKININ RESPONSE FACTORS (*CRFs*) are a small gene family encoding APETALA2 (AP2)/ETHYLENE RESPONSE FACTOR transcription factors, which regulate numerous aspects of plant growth and development (Hallmark and Rashotte, 2019). The Arabidopsis CRF family comprises 12 members characterized by a conserved CRF domain in the N-terminal region, a single AP2 domain for DNA binding, and a C-terminal region (Cutcliffe et al., 2011). *CRFs* respond to phytohormones and environmental stresses including cold (Jeon et al., 2016; Hallmark and Rashotte, 2019). *CRF2* expression is induced by cold and primarily mediated by the cytokinin two-component signaling pathway (Jeon et al., 2016). In contrast, *CRF3* responds to cold independently of the two-component signaling pathway. *CRF2* and *CRF3* regulate lateral root initiation under cold stress.

CRF3 expression was unresponsive to auxin indole-3 acetic acid (IAA) in *arf7 arf19* double mutant in a time-course expression analysis using reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Figure S1A), suggesting that auxin response factor 7 (ARF7)/19 regulate *CRF3* expression in response to auxin. Cold-responsive expression of *CRF3* in *arf7 arf19* was significantly reduced compared with wild-type (WT) after 2 h, whereas a single mutation in *ARF7* or *ARF19* did not alter *CRF3* expression in response to cold (Figures 1A, S1B). Cold-responsive expression of *CRF3* in *arf7 arf19* was similar to WT in response to cold for 2 h, indicating that ARF7 and ARF19 control cold-responsive expression of *CRF3* at later time points (Figure S1B). Combined cold and auxin treatment enhanced *CRF3* expression compared with cold or auxin treatment individually in WT, suggesting an additive effect of these stimuli (Figure S1C). Double mutations in *ARF7* and *ARF19* did not affect cold-responsive expression of *CRF2*, consistent with its auxin-independent regulation (Figure S2). These findings indicated that ARF7/19-dependent and independent pathways control *CRF3* expression in response to cold. Consistent with ARF7/19-regulated expression of *CRF3*, seven auxin-responsive *cis*-acting regulatory elements AuxREs were identified within the *CRF3* promoter region (Figure S3). We also identified five distinct cold-responsive *cis*-acting elements in 10 different

locations, which could be responsible for the early phase of cold-responsive expression of *CRF3* (Figure S4).

Auxin/indole-3acetic acid (Aux/IAA) proteins interact with AUXIN RESPONSE FACTOR (ARF) proteins to repress ARF function (Santner and Estelle, 2009). To test whether mutations in Aux/IAA genes enhance cold-responsive *CRF3* expression by removing the negative regulatory effects of Aux/IAAs, we examined *CRF3* expression in the publicly available *iaa* multiple mutants, the *iaa8 iaa9* double mutant, and the *iaa5 iaa6 iaa19* triple mutant. Although these mutants exhibited neither developmental defects nor significant changes in global gene expression profiles owing to their functional redundancy (Overvoorde et al., 2005), these mutants could serve as sensitized genetic backgrounds to uncover potential roles of Aux/IAA proteins in cold-responsive gene regulation. *CRF3* expression in response to cold was enhanced in *iaa8 iaa9* and significantly enhanced in *iaa5 iaa6 iaa19* compared with the WT (Figure 1B), suggesting that ARF7/19 positively regulated *CRF3* expression in response to cold, whereas Aux/IAA proteins negatively regulated *CRF3* expression.

To determine whether cold could induce the degradation of Aux/IAA proteins, we used transgenic reporter line R2D2 expressing the auxin-dependent degradation domain II (DII) fused to n3 × Venus and a mutated version mDII fused to nuclear-targeted ntdTomato driven by the *RPS5A* promoter (Figure S5A) (Liao et al., 2015). Auxin treatment for 2 h reduced green fluorescence, indicative of DII degradation. However, cold treatment for 4 h had no effect on green fluorescence intensity compared with the untreated reporter plants, suggesting that cold did not induce DII degradation (Figure S5B, C). To assess the effect of cold on ARF7–Aux/IAA interactions, we performed luciferase complementation imaging (LCI) assays in *Nicotiana benthamiana* (Lee et al., 2017). The *N. benthamiana* leaves harboring the indicated constructs were treated at 1°C or 23°C for 4 h. The results showed significantly diminished ARF7–Aux/IAA interactions under cold exposure compared with those under normal temperature, whereas homodimerization of LBD18, used as a negative control, remained unaffected (Figures 1C, S6).

To determine whether the reduced luciferase (LUC) activity observed under cold treatment was due to Aux/IAA protein

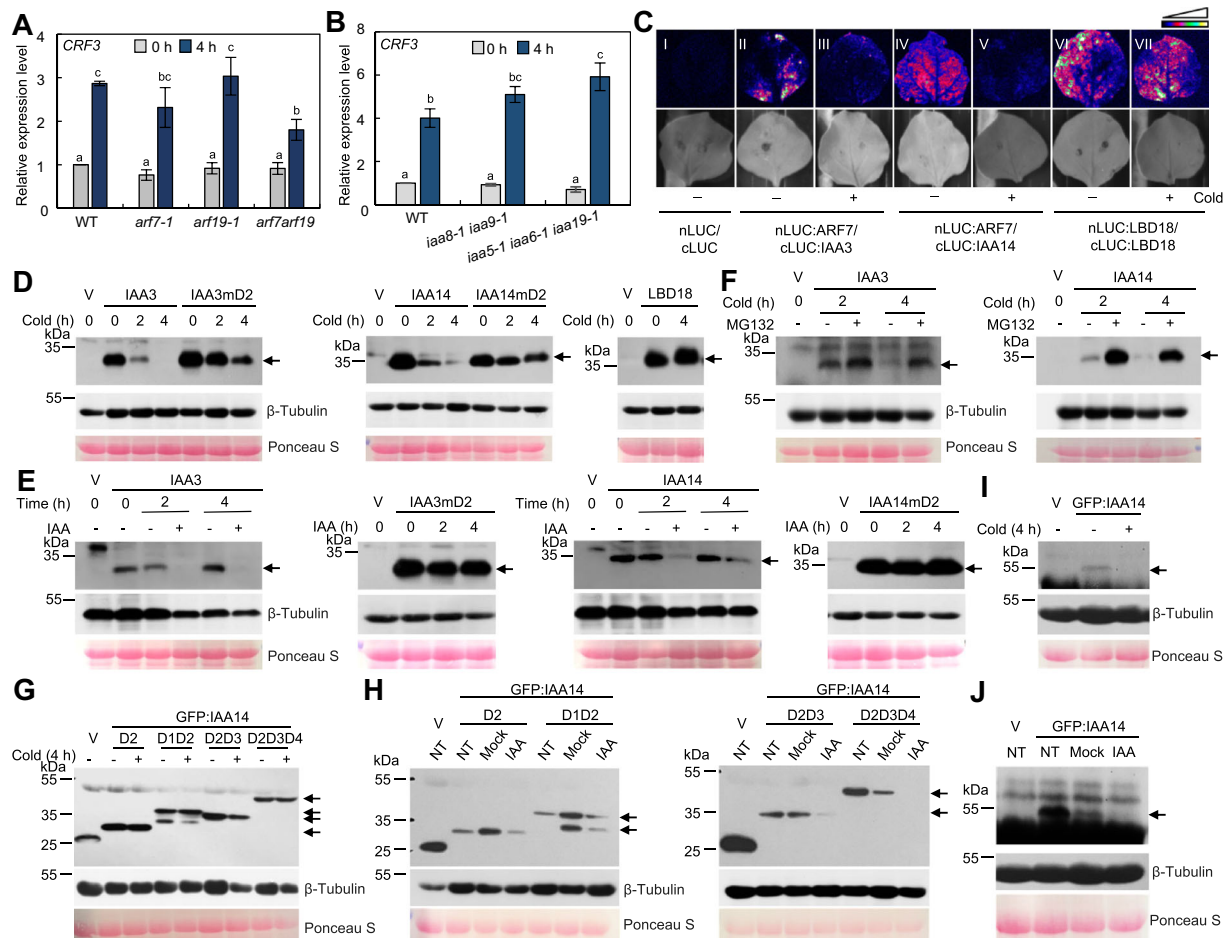


Figure 1. Cold temperature triggers Aux/IAA degradation, releasing ARFs to activate *CRF3* expression

(A) Expression of *CRF3* in wild-type (WT), *arf7* and *arf19* single mutants, and the *arf7 arf19* double mutant in response to cold. Plants at 7-day-old were incubated at 1°C or 23°C for 4 h, and the total RNA isolated was subjected to RT-qPCR. (B) Expression of *CRF3* in WT, *iaa8-1 iaa9-1*, and *iaa5-1 iaa6-1 iaa19-1* mutants in response to cold. Bars with different letters indicate a significant difference at $P < 0.05$ by one-way ANOVA and the Duncan test. (C) LCI assays between ARF7 and Aux/IAA interactions with or without cold exposure at 1°C in *Nicotiana benthamiana* leaves. The pseudo-color bar shows the range of luminescence from weak (blue) to strong (red). (D, E) Analysis of protein stability of IAA3, IAA14, and their DII mutant counterparts in response to cold or IAA in *N. benthamiana* leaves. The *N. benthamiana* leaves harboring each construct were incubated at 1°C (D) or were treated with the buffer containing 20 μM IAA or mock (E), followed by immunoblot analysis using anti-HA antibodies. V, Vector control. The solid arrows indicate IAA protein. (F) Analysis of protein stability of IAA3 and IAA14 in response to cold with or without MG132 treatment in *N. benthamiana*. The *N. benthamiana* leaves harboring each construct were infiltrated with the buffer containing 50 μM MG132 or mock, incubated for 1.5 h, and subjected to cold treatment at 1°C for 2 and 4 h, followed by immunoblot analysis using anti-HA antibodies. (G–J) Analysis of protein stability of IAA14 and IAA14 domains fused with GFP at the N-terminus. The *N. benthamiana* leaves harboring each construct were incubated at 1°C for 4 h (G, I) or infiltrated with 20 μM IAA (H, J), followed by immunoblot analysis using anti-GFP antibodies. D, IAA domain; NT, non-treatment.

degradation, the *N. benthamiana* leaves harboring 3×HA:IAA3 or 3×HA:IAA14 constructs were exposed to 1°C for 0, 2, or 4 h, followed by immunoblot analysis using anti-hemagglutinin (HA) antibodies (Figure 1D). Cold treatment led to degradation of the IAA3 and IAA14 proteins, whereas the DII mutation largely prevented this degradation. In contrast, LBD18 remained unaffected. Degradation of the IAA3 and IAA14 proteins became detectable within 1 and 2 h, respectively (Figure S7), consistent with the ARF7/19-dependent induction of *CRF3* expression in response to cold during a later phase, after 2 h (Figure S1B). When the *N. benthamiana* leaves harboring 3×HA:IAA3 or 3×HA:IAA14 constructs were treated with IAA, both IAA proteins were degraded, whereas the DII mutation completely blocked their degradation (Figure 1E). Tubulin proteins were not affected by either cold or auxin treatment. These findings suggested that

cold stress induces Aux/IAA protein degradation rather than promoting their dissociation from ARF7. MG132, a proteasome inhibitor, suppressed cold-induced degradation of the IAA3 and IAA14 proteins (Figure 1F). To distinguish the identity of the cold-responsive E3 ligase from that of the auxin-responsive E3 ligase, we generated green fluorescent protein (GFP)-tagged IAA14 domain constructs (D2, D1D2, D2D3, D2D3D4, and full length) fused to GFP and examined their stability in *N. benthamiana* leaves after 2 h and 4 h of cold or auxin treatment. All D2-containing proteins were efficiently degraded upon auxin treatment, whereas only the full-length IAA14 protein was destabilized by cold, indicating that cold-induced degradation requires the full structural integrity of IAA14 (Figure 1G–J). Treatment with the auxin antagonist α -(2-oxo-2-phenylethyl)-1H-indole-3-acetic acid (PEO-IAA) blocked auxin-induced, but

not cold-induced, degradation of IAA14 (Figure S8). Cold-induced *CRF3* expression was unaffected in the auxin receptor quadruple mutants (Figure S9). Collectively, these results suggested that cold stress triggers Aux/IAA degradation through a distinct E3 ligase pathway that is likely to operate independently of canonical auxin receptor activity. Distinct E3 ligase(s) may act in concert with cold-responsive signaling components to mediate Aux/IAA protein degradation. Cold-induced degradation of Aux/IAA proteins may represent a regulatory mechanism by which plants modulate auxin signaling to balance growth and developmental processes under cold stress.

ACKNOWLEDGEMENTS

We thank the Arabidopsis Biological Resource Center for *arf7*, *arf19*, and *arf7 arf19* seeds and Dolf Weijers for R2D2 reporter seeds. This study was supported by grants from the National Research Foundation (NRF), funded by the Ministry of Science and ICT of Korea (2021R1A2C1006296 and RS-2024-00344320 to J.K.).

CONFLICTS OF INTEREST

These authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

U.T.N. and N.Y.K. designed and performed the experiments and analyzed the data. D.W.L. analyzed the data and revised the manuscript. J.K. conceived the study, supervised the project, designed the experiments, analyzed the data, and wrote the manuscript with inputs from all authors. All authors have read and approved the contents of this paper.

Edited by: Zhaojun Ding, Shandong University, China

Received Mar. 10, 2025; **Accepted** Aug. 16, 2025; **Published** Sep. 24, 2025

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