Epigenetic switch from repressive to permissive chromatin in response to cold stress

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Edited by Motoaki Seki, RIKEN Center for Sustainable Resource Science, and accepted by Editorial Board Member Caroline Dean May 2, 2018 (received for review December 7, 2017)

Switching from repressed to active status in chromatin regulation is part of the critical responses that plants deploy to survive in an ever-changing environment. We previously reported that HOS15, a WD40-repeat protein, is involved in histone deacetylation and cold tolerance in *Arabidopsis*. However, it remained unknown how HOS15 regulates cold responsive genes to affect cold tolerance. Here, we show that HOS15 interacts with histone deacetylase 2C (HD2C) and both proteins together associate with the promoters of cold-responsive *COR* genes, *COR15A* and *COR47*. Cold induced HD2C degradation is mediated by the CULLIN4 (CUL4)-based E3 ubiquitin ligase complex in which HOS15 acts as a substrate receptor. Interference with the association of HD2C and the *COR* gene promoters by HOS15 correlates with increased acetylation levels of histone H3. HOS15 also interacts with CBF transcription factors to modulate cold-induced binding to the *COR* gene promoters. Our results here demonstrate that cold induces HOS15-mediated chromatin modifications by degrading HD2C. This switches the chromatin structure status and facilitates recruitment of CBFs to the *COR* gene promoters. This is an apparent requirement to acquire cold tolerance.

histone acetylation | derepression | cold stress response | CUL4-based E3 ligase | HOS15

Frosts, particularly at critical stages such as the reproductive development, drop crop yields by ~25% (1, 2). A short exposure to low but nonfreezing temperatures, the so-called cold acclimation, enables plants to tolerate freezing (3, 4). Acclimation involves the stabilization of cellular membranes, enhancement of antioxidative stress mechanisms, and accumulation of cryoprotectants (5). Low temperature initiates signaling cascades regulating expression of genes involved in cold stress response or tolerance (6, 7) and cold acclimation (1, 4, 8, 9). During the last decade, regulators and effectors of cold signaling, and numerous output genes have been identified (3, 10, 11). The best-characterized transcription factors belong to the C-REPEAT (*CRT*) BINDING FACTORS (CBFs)/DEHYDRATION RESPONSIVE ELEMENT (*DRE*) BINDING FACTORS (DREBs) family. CBF/DREB transcription factors control cold-dependent and ABA-independent expression of COLD RESPONSIVE (*COR*)/RESPONSIVE TO DESICCATION (*RD*)/LOW-TEMPERATURE–INDUCED (*LTI*)/*KIN* (stress-induced) genes through association to *CRT/DRE cis*-elements that are also found in their own promoters (4, 12, 13). Overexpression of *CBF1*, *-2*, or *-3* induces the expression of CBF regulons and enhances freezing tolerance (10, 14), whereas *cbf1/2/3* triple mutants exhibit extreme freezing sensitivity (15, 16), indicating that *CBFs* have a critical role in freezing tolerance in *Arabidopsis*.

Regulation of gene expression in response to cold stress often employs posttranslational histone modifications, including histone acetylation, methylation, and phosphorylation (17, 18). Acetylation and deacetylation of lysine residues at the N terminus of histones, which are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, have especially been extensively shown to be involved in abiotic stress responses (17–21). Acetylation of lysine residues of histones H2B, H3, and H4 neutralize their positive charges, reducing the strength of the interaction with DNA and inducing an "open" chromatin configuration that correlates with transcriptional activation, whereas histone deacetylation induces a "closed" compact chromatin state and is linked with transcriptional repression (18, 22, 23). HATs often interact with various chromatin remodeling proteins to form transcriptional coactivator complexes, which recognize histone marks and modify chromatin, and recruit transcription factors to the target chromatin regions for gene induction (24). Oppositely, many transcriptional corepressors are known to associate with HDAC (25–27). Despite these insights, the contribution of histone modification and epigenetic regulation to plant stress tolerance remains unclear.

Significance

Phenotypic adaptations of plants in response to changes in climate are well known to be mediated by molecular mechanisms, including activation or suppression of transcription factors that control target gene expression. However, the chromatin changes that are essential for the binding of transcription factors are much less understood. Gene derepression at the chromatin level is considered to be the starting point for gene transcription. We report a mechanism of gene derepression through which HOS15 promotes the degradation of histone deacetylase HD2C in a cold-dependent manner that correlates with increased levels of acetylated histones on *COR* gene chromatin. Moreover, HOS15 directly promotes *COR* gene transcription by association of CBF transcription factors with the "open" state of the target *COR* chromatin.

Author contributions: J.P., C.J.L., M.S., and D.-J.Y. designed research; J.P., C.J.L., M.S., J.-Y.C., E.I., and V.R. performed research; J.P., C.J.L., M.S., H.J.P., T.M., J.-K.Z., R.A.B., S.Y.L., J.B.J., W.-Y.K., and D.-J.Y. analyzed data; and J.P., V.R., B.-h.L., J.M.P., and D.-J.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.S. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1721241115/-/DCSupplemental.

Published online May 21, 2018.

Posttranslational covalent modification of proteins causes rapid and reversible/irreversible alterations in their function. For example, conjugation of ubiquitin (Ub) to substrate proteins (ubiquitination) generally implies that the target proteins are subjected to proteasome degradation, which has substantial effects on regulatory processes including transcription (28, 29). In the Ub–proteasome system, Ub attachment to a target substrate involves sequential steps referred to as activation (E1), conjugation (E2), and ligation (E3) (30). CULLIN RING ligases (CRLs) are the largest family of E3 Ub-ligases in *Arabidopsis*. Among the CRLs, the scaffold protein CULLIN4 (CUL4) assembles a small RING-box domain protein (RBX1) on its C terminus and the DNA BINDING PROTEIN 1 (DDB1) on its N terminus to interact with substrate receptors, namely DCAFs (CUL4- and DDB1-associated factors), that recognize their corresponding targets for ubiquitination. The DCAFs usually possess the DDB1-binding WD40 protein (DWD) domain, which consists of 16 amino acids within WD40 repeats and are conserved in many eukaryotes (31–34). In *Arabidopsis*, 85 proteins are found to contain the DWD motif and have diverse functions in regulation of development and stress responses (33, 35, 36).

Previously, we reported that the WD40-repeat protein HOS15 is involved in histone modification and cold tolerance in *Arabidopsis* (37). However, few mechanistic links between the regulation of cold stress response and chromatin dynamics have been identified in plants, and how HOS15 is involved in gene expression through chromatin remodeling to regulate cold adaptation remains unknown. In this work, we demonstrate that HOS15 functions as a DCAF protein and leads to the ubiquitination and degradation of HISTONE DEACETYLASE 2C (HD2C), thereby modulating chromatin status and gene expression of *COR* genes in response to freezing stress in *Arabidopsis*. Our findings provide insights into how chromatin remodeling is linked with cold stress responses in plants.

Results

HOS15 Interacts with HD2C in Nuclei. To identify interacting proteins working together with HOS15, especially in gene-expression control, we carried out a yeast two-hybrid screening. The full-length cDNA of *HOS15* and an *Arabidopsis* cDNA library obtained from the *Arabidopsis* Biological Resource Center (The Ohio State University, Columbus) (38) were cloned into bait and prey plasmids, respectively. Fifty-four clones survived on the stringent media (-TLH) and seven putative HOS15-interacting partners including HD2C were identified (Fig. 1*A* and *SI Appendix*, Table S1). As we were more interested in how HOS15 is involved in epigenetic regulation, the interaction of HOS15 and HD2C (NM_120455.3, At5g03740) (39, 40) was confirmed using coimmunoprecipitation (co-IP) (Fig. 1*B*) (37). Total protein extracts from tobacco plants transiently expressing HOS15-FLAG and HD2C-GFP were pulled down with anti-FLAG and HD2C-GFP was detected using anti-GFP (Fig. 1*B*). The interaction of HOS15 with HD2C was further tested by using a split-luciferase (LUC) complementation assay, which is based on the reconstituted LUC activity when two proteins respectively fused with N- and C-terminal LUC fragments (NLuc and CLuc) physically interact in vivo (41, 42). Coexpression of *CLuc-HOS15* and *HD2C-NLuc* in tobacco leaves resulted in high luminance signals (Fig. 1*C* and *SI Appendix*, Fig. S1), revealing physical interaction between HOS15 and HD2C. Consistent with the known localization of HOS15 in nuclei (37), YFP^{N}-HOS15 expressed in *Arabidopsis* protoplasts interacted with YFP^{C}-HD2C in the nucleus as shown by biomolecular fluorescence complementation (BiFC) assays (Fig. 1*D*).

HOS15 and HD2C Are Involved in Freezing Stress Responses. As the *hos15-1* allele is in the C24 ecotype background (37) and *hos15-2* (GK_785B10) is in Col-0 background, the complementation lines *HOS15pro::HOS15-HA* were generated in *hos15-2* (*SI Appendix*, Fig. S2 *A–E*). We also obtained two mutant alleles of *HD2C*, *hd2c-1* (SALK_129799) and *hd2c-3* (SALK_039784), and transgenic plants overexpressing *HD2C-GFP* (*HD2Cox*) (40, 43, 44) (*SI Appendix*, Fig. S3 *A–E*). The *hos15-2* loss-of-function mutant exhibited cold sensitive phenotypes, with and

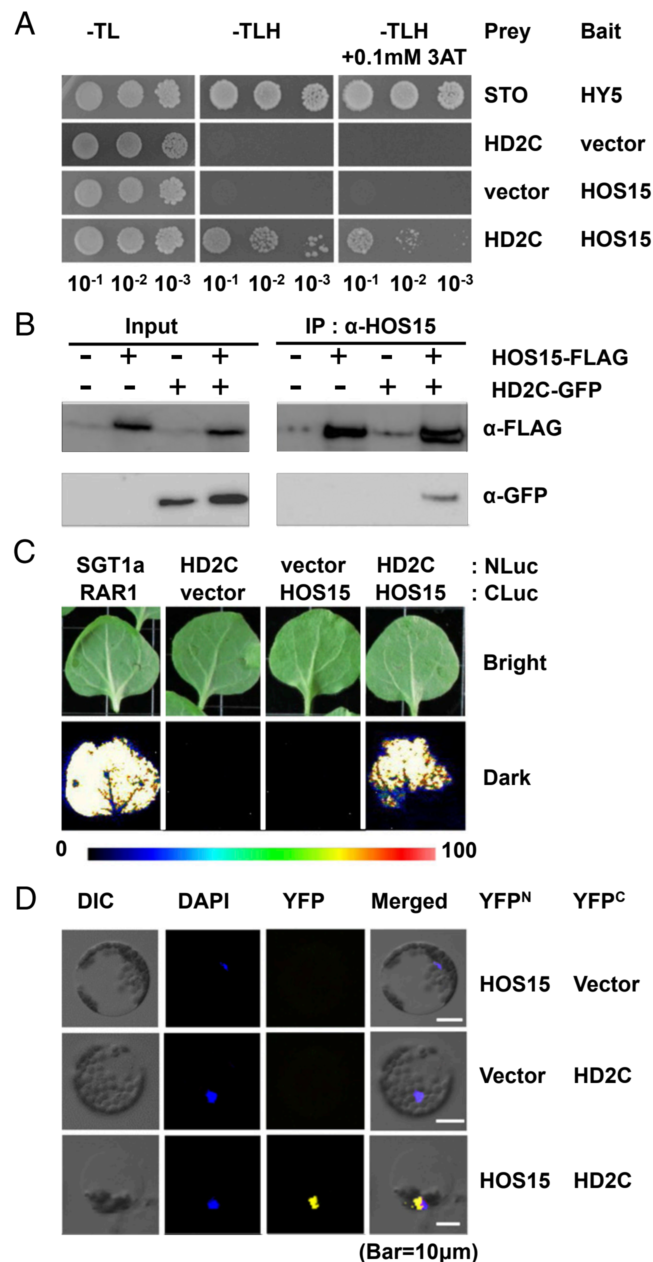

Fig. 1. HOS15 directly interacts with HD2C. (*A*) HOS15 interacts with HD2C by yeast two-hybrid assay with HOS15 and HD2C as bait and prey, respectively. (*B*) HOS15 binds to HD2C in vivo. Tobacco plants were infiltrated with *Agrobacterium* harboring *35S::HOS15-FLAG* and *35S::HD2C-GFP* for transient expression. Protein extracts (input) were immunoprecipitated (IP) with anti-FLAG, and resolved by SDS/PAGE. Immunoblots were developed with anti-FLAG and anti-GFP to detect HOS15 and HD2C fusions, respectively. (*C*) HOS15 interacts with HD2C in vivo. *HOS15* and *HD2C* were fused to C terminal of *CLuc* and N terminal of *NLuc* for split luciferase complementation assays. *Agrobacterium* carrying *35S::CLuc-HOS15* and *35S::HD2C-NLuc* were infiltrated into tobacco leaves for transient expression. After 2 to 3 d, the bottom side of the tobacco leaves were sprayed with 1 mM luciferin, and bioluminiscence was detected. Combination of *CLuc-RAR1* and *SGT1a-NLuc* is included as a positive control. Images shown are representative of three biological replicates of three individual experiments. (*D*) HOS15 interacts with HD2C in vivo. Shown are the results of BiFC analyses performed with constructs of *VYNE-HOS15* and *VYCE-HD2C*, which were transiently expressed in *Arabidopsis* protoplasts. Nuclei were stained with DAPI, and YFP fluorescence was detected under the confocal microscope. (Scale bars, 10 μm.)

without cold-acclimation (Fig. 2 *A–C* and *SI Appendix*, Fig. S2 *F–I*), as previously observed in the *hos15-1* mutant (37). Both visual assessment and an electrolyte leakage assay revealed that freezing tolerance levels in *hd2c* mutants were comparable to those in the wild-type without cold-acclimation and even better than in the wild-type upon cold acclimation (Fig. 2 *A–C* and *SI Appendix*, Fig. S3*F*). However, *HD2Cox* plants showed sensitivity to freezing stress compared with the wild-type (Fig. 2 *A–C* and *SI Appendix*, Fig. S3 *G–I*). These results suggest that HD2C, a plant-specific histone H3 deacetylase (44), is negatively involved in cold stress signaling.

As low temperatures transiently induce expression of *CBF* and CBF-regulated *COR* genes (45, 46), transcript levels of *CBFs* and *CORs* in wild-type, *hos15-2*, *hd2c-1*, and *HD2Cox* plants were checked (Fig. 2*D* and *SI Appendix*, Fig. S4*A*). Consistent with previous reports (47), transcripts of *CORs*, including *COR15A*, *COR47*, and *RD29A*, began to accumulate in the wild-type after 6–12 h upon exposure to cold (Fig. 2*D*). Transcript levels were significantly reduced in *hos15-2* and *HD2Cox*, whereas they were substantially higher in *hd2c-1* than in the wild-type upon cold treatment (Fig. 2*D*). However, cold-induced expression of *CBF* genes and accumulation of their protein product were similar in wild-type, *hos15-2*, *hd2c-1*, and *HD2Cox* plants (*SI Appendix*, Fig. S4),

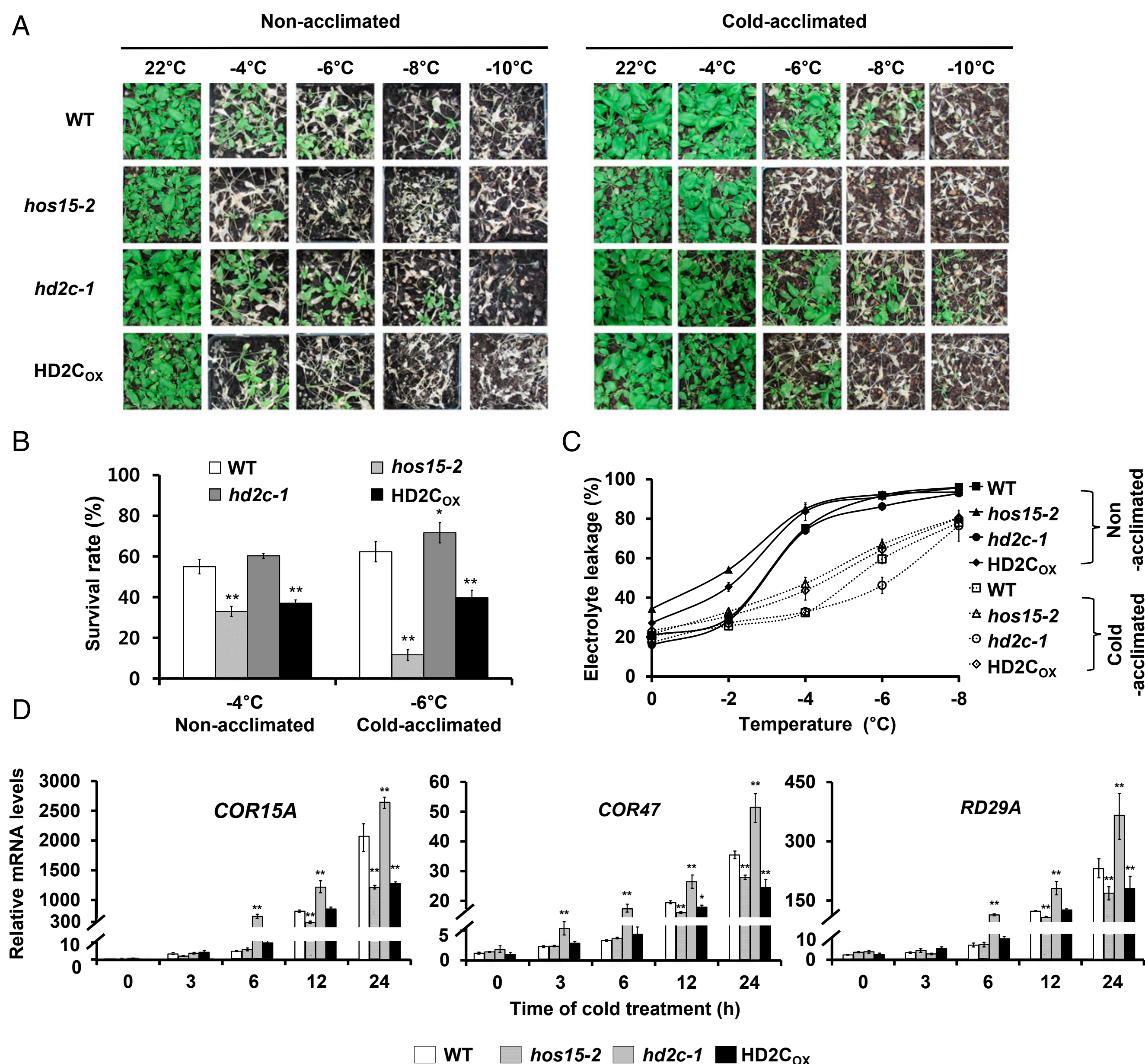

Fig. 2. HD2C is involved in freezing stress response. (*A–C*) *hd2c* is tolerant to freezing stresses. Three-week-old plants pretreated with cold (4 °C for cold-acclimation) or not (nonacclimation) were exposed to freezing temperatures as indicated. (*B*) Survival ratio was determined with nonacclimated (–4 °C) or cold-acclimated (–6 °C) plants in 7 d after freezing treatment. The data are the means of three technical replicates with SD (*n* = 25 for each replicate: **P* < 0.05; ***P* < 0.01; Student's *t* test). (*C*) Electrolyte leakages of nonacclimated (*A*, *Left*) or acclimated (*A*, *Right*) plants were measured at indicated temperatures. Error bars are SD (*n* = 6). (*D*) Relative transcript levels of cold responsive genes are higher in *hd2c* mutant upon cold treatment. Two-week-old plants including wild-type (white bar) or *hos15-2* (hatched bar) and *hd2c-1* (gray bars) or $HD2C_{OX}$ (black bars) were treated with cold (0 °C) for indicated periods. Total RNA was isolated and transcript levels of *CORs* were measured by qRT-PCR and normalized to that of *ACTIN2*. Bar represent means ± SD from three biological replicates with three technical repeats each.

suggesting that HOS15 and HD2C influence the expression of *COR* genes by another mechanism other than controls on *CBF* transcription or CBF protein abundance.

HOS15 Is Part of CUL4-Based E3 Ub Ligase Complexes. Proteins containing a DWD motif act as receptors for CUL4-based E3 ligases (CRL4), where they help to recruit specific substrates for degradation in both plant and animal systems (31–33, 48). HOS15 is predicted to contain a conserved DWD motif within the third WD40 repeat, between amino acids 380 and 395 (33, 37). Indeed, HOS15 directly bound DDB1B, a component of CRL4 complexes, in yeast two-hybrid assays (Fig. 3*A*). The interaction of HOS15 and DDB1 was further confirmed *in planta* by co-IP assays of HOS15-FLAG with DDB1A-HA or DDB1B-HA transiently expressed in tobacco leaves. When HOS15-FLAG from total protein extracts was pulled down, DDB1A-HA and DDB1B-HA were detected to interact with HOS15-FLAG (Fig. 3 *B* and *C*). In addition, HOS15 associated with CUL4, the scaffold component in CRL4 complexes (Fig. 3*D*), and such interaction was enhanced upon cold treatment in *Arabidopsis* (Fig. 3*E*), suggesting that HOS15 assembles into CRL4 E3 Ub ligase complexes (henceforth $CRL4^{HOS15}$).

Because HOS15 interacted with CRL4 components, we tested whether HD2C also associates to CUL4 and DDB1B using co-IP (*SI Appendix*, Fig. S6*D*). Total protein extracts from wild-type, *hd2c-1*, *FLAG-CUL4* overexpressor (*FLAG-CUL4*), and *FLAG-DDB1B/ddb1a* (*FLAG-DDB1B*) plants were immunoprecipitated with anti-HD2C antibody, and both DDB1B and CUL4 fusions were found to coprecipitate with HD2C, suggesting that HD2C also associates to $CRL4^{HOS15}$ E3 ligase complexes, likely as a target.

HOS15 Is Required for Cold-Induced Ubiquitination and Degradation of HD2C. DWD proteins act as substrate receptors within CRL4 complexes, facilitating ubiquitination and subsequent degradation of specific protein targets through the Ub–proteasome system (33, 35). According to this notion, we tested whether HD2C serves as a substrate of the $CRL4^{HOS15}$ complex and is subjected to ubiquitination and proteasome-mediated degradation. Indeed, the abundance of HD2C-GFP protein in *HD2C* overexpressing plants (*HD2Cox*) and of the native HD2C protein in wild-type plants gradually decreased upon cold treatment with minor changes of *HD2C* mRNA abundance (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S5). Disappearance of GFP fluorescence from *HD2C-GFP* overexpressing plants was also promoted by cold treatment (Fig. 4*C*). Furthermore, treatment with the proteasome inhibitor MG132 during cold treatment abolished the cold-induced decrease in the steady-state levels of HD2C protein (Fig. 4*D*), suggesting that cold-induced degradation of HD2C proteins is mediated by the proteasome complex. However, the cold-induced degradation of HD2C was impaired in *hos15-2* and *cul4-1* mutants (Fig. 4*D* and *SI Appendix*, Fig. S6*E*), and *CUL4* defective plants were sensitive to freezing (*SI Appendix*, Fig. S6 *A*–*C*), strongly suggesting that cold-induced HD2C destabilization is mediated by HOS15 and CUL4.

As the cold-induced reduction of HD2C protein abundance likely results from Ub degradation, Ub-conjugated proteins were purified from cold-treated *HD2C-GFP* overexpressing plants in wild-type and *hos15-2* backgrounds using commercially available p62 resin that binds Ub noncovalently (Fig. 4*E*). The p62 affinity-purified samples showed extensive ubiquitination, as detected in immunoblots with antiubiquitin antibody (Fig. 4*E*, *Left*). Immunoblots of the affinity purified extracts from *HD2C-GFP*–overexpressing plants (*HD2Cox*) using anti-GFP antibody showed the presence of HD2C-GFP as multiple high molecular mass bands, while no signal was detectable in the *hos15-2* mutant. These results indicate that HD2C-GFP is modified with poly-ubiquitin chains, that HOS15 is required for cold-induced poly-ubiquitination of HD2C (Fig. 4*E*, *Right*), and imply that cold sensitivity of *hos15-2* results from the failure of HOS15-mediated HD2C degradation. Indeed, *hos15-2 hd2c-1* double-mutant plants suppressed the cold-sensitive phenotypes of *hos15-2* (*SI Appendix*, Fig. S7 *A*–*C*). Furthermore, the

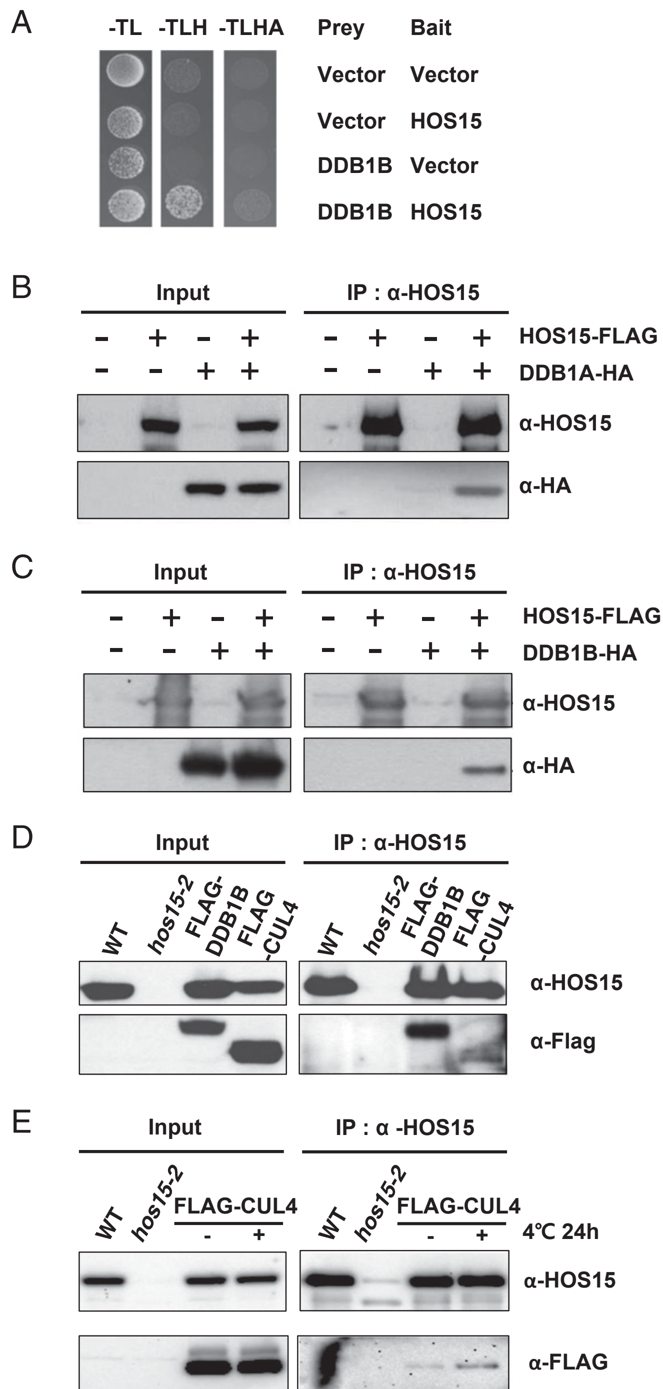

Fig. 3. HOS15 is a component of CUL4-based Ub E3 ligase complexes. (*A*) HOS15 interacts with DDB1 directly in yeast two-hybrid assays. Assays were performed with DDB1B protein as prey and HOS15 as bait. (*B* and *C*) HOS15 interacts with DDB1 proteins in vivo. Total proteins (input) extracted from tobacco plants transiently expressing HOS15-FLAG and DDB1A-HA (*B*) or DDB1B-HA (*C*) were immunoprecipitated (IP) with anti-HOS15. Immunoblots were carried out with anti-HOS15 and anti-HA to detect HOS15-FLAG and DDB1A-HA or DDB1B-HA, respectively. (*D*) HOS15 interacts with CUL4 in vivo. Co-IP of HOS15 and DDB1B or CUL4. Total proteins from 12-d-old *35S::Flag-DDB1B/ddb1a* and *35S::Flag-CUL4* plants were pulled down with anti-HOS15. Anti-FLAG was used to detect DDB1B and CUL4. (*E*) Cold enhances the interaction of HOS15 and CUL4. Total protein extracts from *CUL4* overexpressors exposed to cold stress (4 °C) for 24 h were pulled down with anti-HOS15.

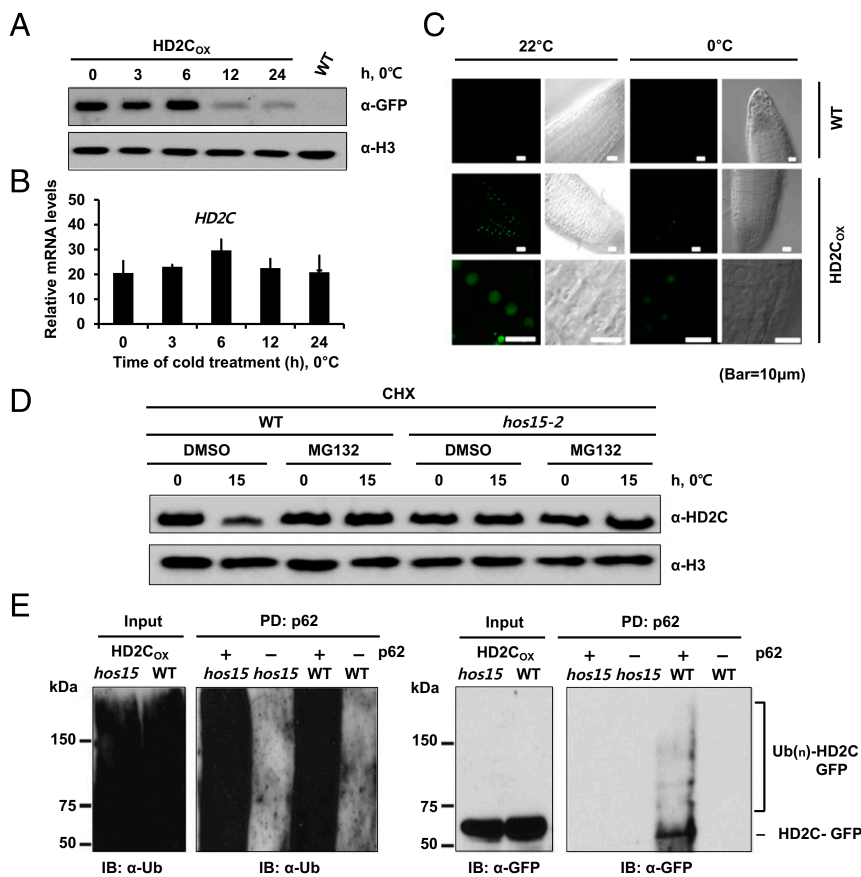

Fig. 4. Cold-induced degradation of HD2C is mediated by HOS15. (*A*) The protein abundance of HD2C is reduced upon cold stress. Nuclear proteins extracted from 12-d-old *HD2C* overexpressors treated with cold stress (0 °C) for the indicated periods were applied to immunoblots with anti-GFP. Hintone3 (H3) was used as loading controls. (*B*) Relative *HD2C* mRNA levels in *HD2C* overexpressors are not reduced upon cold. Total RNA was extracted and gene expression of selected genes was checked by qRT-PCR analysis. Results are from three biological replicates and values represent means ± SD (*n*= 9). (*C*) Cold reduces the biofluorescence from 7-d-old *HD2C-GFP* overexpressing plants exposed to cold (0 °C) for 6 h. (*D*) Cold-induced degradation of HD2C is impaired in *hos15-2*. Ten-day-old wild-type and *hos15-2* plants were treated with cold (0 °C) for 15 h in the presence of proteasome inhibitor MG132 (50 μM). Nuclear proteins were applied to immunoblot with anti-HD2C. (*E*) The poly-ubiquitination of HD2C is blocked in *hos15-2* mutant. Seven-day-old plants (*HD2C-GFP* and *hos15-2 HD2C-GFP*) were treated with cold (4 °C) for 12 h in the presence of MG132. Total proteins were incubated with Ub-binding p62 resin or with empty agarose resin (negative control). Anti-Ub was used to detect ubiquitinated proteins in input protein extracts and pulled-down (PD) samples. Anti-GFP allowed the detection of HD2C-GFP and its ubiquitinated forms Ub (n)-HD2C-GFP.

reduced expression of *COR* genes in *hos15-2* was also suppressed by *hd2c-1* (*SI Appendix*, Fig. S7*D*). Taken together, these observations are evidence that HOS15-dependent ubiquitination and degradation of HD2C contributes to plant tolerance to cold.

Association of HOS15 and HD2C to Promoter Regions of *COR* Genes Is Altered by Cold Stress. Next, to analyze whether the HOS15–HD2C HDAC complex associates to chromatin at *COR* genes, chromatin immunoprecipitation (ChIP) assays were performed. Under ambient temperature, HOS15 and HD2C proteins were found to bind the promoter of *COR15A* and *COR47* genes, mainly in regions containing a *CRT*/*DRE* element where CBF proteins bind (Fig. 5 *C*–*F* and *SI Appendix*, Fig. S8). The binding of HOS15 to these regions was enhanced upon cold treatment while association of HD2C to the identical regions of *COR15A* and *COR47* where HOS15 binds was dramatically decreased (Fig. 5 *E* and *F* and *SI Appendix*, Fig. S8). Presumably, attenuation of the association of HD2C to *COR* gene chromatin results from cold-induced degradation of HD2C mediated by HOS15.

HD2C is known to bind to and deacetylate histone H3 (44, 49). Because HOS15 interacts with HD2C and regulates *COR* gene expression (Figs. 1 and 2), we investigated how HOS15 is functionally linked with HD2C in terms of chromatin regulation of *COR* genes expression. In wild-type plants, H3 acetylation (AcH3) level on *COR15A* promoter regions bound by CBF proteins was significantly increased by cold treatment. However, *hd2c-1* plants displayed high accumulation of AcH3 on the *COR15A* promoter with or without cold treatment (Fig. 5*B*). In *hos15-2* plants, cold treatment failed to induce the acetylation of H3 on *COR15A*. The accumulation of AcH3 in *hos15-2 hd2c-1* double mutants regardless of temperature supported that *hd2c-1* is epistatic to *hos15-2*, and HOS15-mediated transcriptional regulation of *COR* genes expression in response to cold stress is at least partially mediated by HD2C. Furthermore, the association of HD2C to the *CRT*/*DRE* regions of *COR15A* chromatin was lost in *hos15-2* at normal temperature (Fig. 5*H*) (22 °C), indicating that HOS15 is required for the efficient binding of HD2C to the promoter of *COR* genes. Upon cold stress, the amount of HD2C in the *COR15A* chromatin in *hos15-2* plants was still low despite its greater stability in the absence of HOS15 (Fig. 5*H*) (0 °C), and was reduced further in the wild-type, as expected from the cold-induced HD2C degradation mediated by HOS15. On the other hand, association of HOS15 to the *CRT*/*DRE* regions of *COR15A* chromatin was the same in wild-type and *hd2c-1* at normal temperature (Fig. 5*G*) (22 °C). However, the increased binding of HOS15 to *COR15A* chromatin upon cold stress was not observed in the *hd2c-1* mutant (Fig. 5*G*) (0 °C).

HOS15 Promotes the Binding of CBF Proteins to *COR* Promoter Regions. We have shown that HOS15 and HD2C associate to *CRT*/*DRE* regions of *COR* genes where CBF proteins bind to enhance *COR* gene expression (Fig. 5 and *SI Appendix*, Fig. S8). In fact, yeast two-hybrid, co-IP, and split-LUC complementation assays showed that HOS15 interacted with all CBF isoforms (Fig. 6*A* and *SI Appendix*, Fig. S9 *A* and *B*). In contrast, HD2C failed to interact with all CBFs (*SI Appendix*, Fig. S9*C*) Accordingly, gel-filtration assays followed by western blotting showed that CBF proteins from cold-stressed plants (0 °C, 24 h) were detected in fractions corresponding to complexes ranging from approximately 200–660 kDa, which overlaps with the molecular mass range of HOS15 complexes (200–660 kDa) (Fig. 6*B*). Thus, we examined whether HOS15 and HD2C affected the binding of CBF proteins to *CORs* promoters in response to cold stress (Fig. 6 *C* and *D* and *SI Appendix*, Fig. S10). Cold (0 °C, 24 h) greatly enhanced the binding of CBF proteins to the *CRT*/*DRE* regions of *COR15A* and *COR47* in the wild-type. However, CBF binding induced by cold treatment was dramatically reduced in *hos15-2* plants, indicating that HOS15 facilitates the binding of CBF

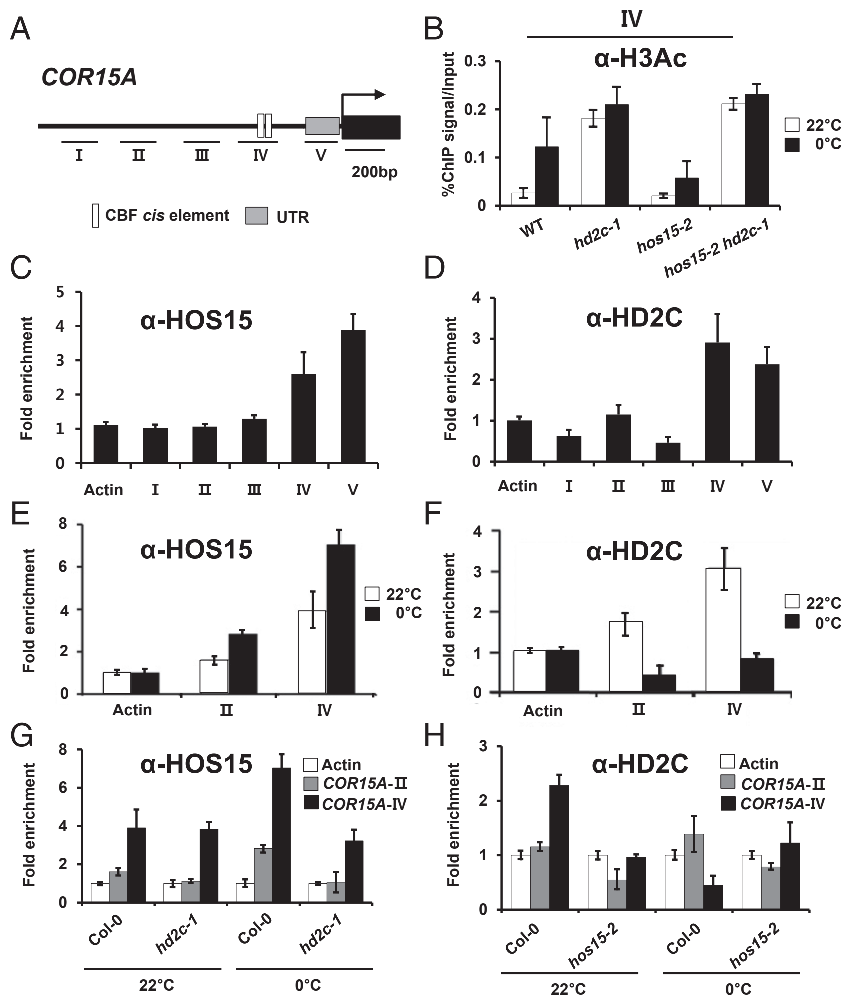

Fig. 5. HD2C and HOS15 associate to the promoter locus of *COR15A*. (*A*) Structure of the *COR15A* promoter and amplicon regions (I to V) used for ChIP. The arrow indicates the TSS. White boxes mean CBF binding *cis* elements, and gray box denotes 5′UTRs. (*B*) Cold-induced H3 acetylation of the *COR15A* promoter locus is impaired in *hd2c* mutants. Chromatin from wild-type, *hd2c-1*, *hos15-2*, and *hos15-2hd2c-1* plants treated with cold (0 °C) for 24 h were immunoprecipitated with anti-H3Ac antibody. A control reaction was processed in parallel with rabbit IgG. ChIP and input-DNA were applied to real-time qPCR using primers specifically targeting to the *COR15A* promoter region, IV. Error bars indicate SE (*n* = 3). The experiments were repeated two times with similar results. (*C* and *D*) HOS15 and HD2C associates to regions IV and V of the *COR15A* promoter, CBF *cis* element regions. Chromatin complexes from wild-type plants were immunoprecipitated with anti-HOS15 (*C*) or anti-HD2C (*D*). A control reaction was processed in parallel with rabbit IgG. ChIP and input-DNA samples were quantified by real-time qPCR using primers specific to the different regions (I to V) of the *COR15A*. (*E* and *F*) Cold enhances the binding of HOS15 protein to *COR15A* promoter but reduces that of HD2C protein. ChIP assay was carried out using anti-HOS15 (*E*) and anti-HD2C (*F*) with wild-type plants treated with cold (0 °C, for 24 h). (*G* and *H*) HOS15 is required for HD2C association to *COR* genes chromatin and vice versa. Four-week-old seedling (22 °C) or 4-wk-old seeding after cold (0 °C) treatment 1 d were used for isolating input chromatin. ChIP data from wild-type and *hos15-2* or hd2c-1 plants. Epitope-tagged HD2C chromatin complex was immunoprecipitated with anti-HD2C antibody or HOS15 chromatin complex was immunoprecipitated with anti-HOS15. A control reaction was processed in parallel with rabbit IgG. ChIP and input-DNA samples were quantified by real-time qPCR using promoter specific to the different region of the *COR15A* genes. The structures of the *COR15A* gene as well as the position of the primer used for qRT-PCR corresponding to the distal promoter regions are marked on the diagram at the top. The ChIP results are presented as fold-enrichment of nontarget DNA. Error bars indicate SE (*n* = 3). The experiments were repeated at least two times with similar results.

proteins to the chromatin of *CORs*. Moreover, the association of CBF proteins to *COR* promoters was significantly enhanced in cold-treated *hd2c-1* compared with wild-type, suggesting that removal of HD2C by HOS15 is required for CBFs-binding to *CRT*/*DRE* regions of the *COR* chromatin in response to cold stress. These results indicate that HOS15 interacts with CBFs during cold stress and that this complex positively regulates *COR* gene expression in response to cold stress. Furthermore, we have tested the association of HD2C and HOS15 to the CRT/DRE element in the *cbf1*/*2*/*3* triple mutant to analyze whether the binding of HOS15 and HD2C is dependent on CBFs. As shown in Fig. 6 *E* and *F*, the recruitment of HOS15 and HD2C was greatly reduced in the *cbf1*/*2*/*3* triple mutant, regardless of the temperature. Of note is that CBFs have a low but detectable level of expression at 22 °C (*SI Appendix*, Fig. S4) and that the basal association of HOS15 to the *COR15A* promoter at 22 °C disappeared in the *cbf1*/*2*/*3* mutant. These results indicate that CBFs are bound to CRT/DRE elements even at regular growth temperature and that upon their own induction by cold stress they help recruiting HOS15 to *COR* chromatin.

Discussion

HOS15 is a homolog of human TBL1X (transducin β-like 1 X-linked) and TBL1XR1 (transducin β-like 1 X-linked receptor 1) that are core components of nuclear receptor corepressor (N-CoR), also known as SMRT (silencing mediator for retinoid and thyroid hormone receptors) corepressor complex (50). This corepressor complex recruits HDAC3 to gene promoter regions (51). The *Arabidopsis* N-CoR homolog PWR (*powerdress*) interacts with HDA9 and promotes histone H3 deacetylation (52, 53), and we have shown that the corepressor complex in *Arabidopsis* contains HOS15, which interacts with several histone deacetylases including HDA9 (*SI Appendix*, Fig. S1). These data indicate that the plant version of N-CoR/SMRT complex includes similar components as in animals. Moreover, our split luciferase assay showed that HOS15 makes a complex with class I type HDACs (HDA6, HDA9, HDA19) and plant-specific HD2 type HDACs (HD2A, HD2B, HD2C) (*SI Appendix*, Fig. S1), which expands further the structural analogy to the animal system.

N-CoR/SMRT corepressor complexes have been well studied in association with nuclear hormone receptors (54). For transcriptional repression, N-CoR/SMRT repressor complexes are recruited to ligand-unbound retinoic acid and thyroid hormone receptors that bind to response elements in target genes (25). Upon ligand binding, TBL1X and TBL1XR1 are activated and serve as E3 Ub ligase receptors for the recruitment of the ubiquitination machinery and, eventually, for proteasome-dependent degradation of the N-CoR/SMRT corepressor complexes. Our observations are aligned to this general mechanism because HOS15 interacted with DDB1B and CUL4 (Fig. 3), suggesting that HOS15 is a component of CUL4-based Ub ligase complexes. Upon cold signaling, HOS15-containing Ub ligase complex showed ubiquitination activity on HD2C (Fig. 4), resulting in its proteasome-mediated degradation. How would the cold signal be transmitted to HOS15 for *COR* genes regulation? Our ChIP assays indicated that association of HOS15 to *CRT*/*DRE* elements increased during cold stress. This could be achieved, at least in part, through the interaction with cold-induced CBFs that specifically bind to *COR* promoters. Still, the question remains

whether a posttranslational modification (PTM) or intrinsic property of HOS15 elicits ubiquitination of HD2C upon cold-sensing. Many signal transduction pathways involve PTM on target proteins (55). Indeed, TBL1X and TBL1XR1 (the HOS15 homologs) are regulated by phosphorylation and sumoylation during nuclear receptor ligand and Wnt-protein signaling, respectively (50, 56). HOS15 contains multiple putative phosphorylation and sumoylation sites identified by NetPhos (www.cbs.dtu.dk/services/NetPhos/) and SUMOplot (www.abgent.com/sumoplot). However, our immunoblot analyses of HOS15 did not show up-shifted band patterns indicative of PTMs after cold treatment (Fig. 3). However, protein homodimerization acts as an activator of polyubiquitination of the target protein by the SCF complex (57). Sequence analysis revealed that of HOS15 contains a LisH domain potentially involved in homodimerization. Together, these results suggest that HOS15 could dimerize under cold stress and activate CRL4 to degrade HD2C. Further studies are needed to test our hypothesis.

HOS15 does not contain a DNA binding domain and N-CoR/SMRT corepressor complexes also do not directly bind to the *cis* element in the target gene promoters. However, HOS15 and HD2C strongly associated to the CTR/DRE elements at *COR* gene promoters, which are the binding element for CBF transcription factors. Binding of human N-CoR/SMRT complexes to their response elements is mediated by nuclear hormone receptors that stay bound in the presence and absence of ligands. Thus, through ubiquitination and degradation of the complex, TBL1X and TBL1XR1 facilitate a switching process between coactivator and corepressor complexes on the target chromatin, where TBL1X and TBL1XR1 serve as a platform for this exchange. HOS15 interacts with CBFs, and these transcription factors have a low but detectable expression level (*SI Appendix*, Fig. S4). Although *CBF* genes are highly induced by low temperatures, the expression of *CBF* genes appeared to oscillate at ambient temperature, following a circadian rhythm (58). Additionally, the *cbf1/2/3* triple knockout mutant was smaller in size than the controls (16), suggesting a constitutive housekeeping role for CBF transcription factors under normal conditions. Because the abundance of HOS15 in *COR15A* chromatin was low in the *cbf1/2/3* mutant regardless of the temperature (Fig. 6 *E* and *F*), it is tempting to speculate that CBF proteins could be already present on the *COR* gene promoters even under ambient temperature conditions, helping to recruit HOS15 and HD2C, and the rest of the corepressor complex, to the CBF binding element for *COR* gene repression. Thus, similarly to TBL1X and TBL1XR1, HOS15 could function as an exchange factor or a platform protein, as HOS15 appeared to stay very strongly associated to CBF-binding elements in the *COR* gene promoters even after HD2C was mostly removed

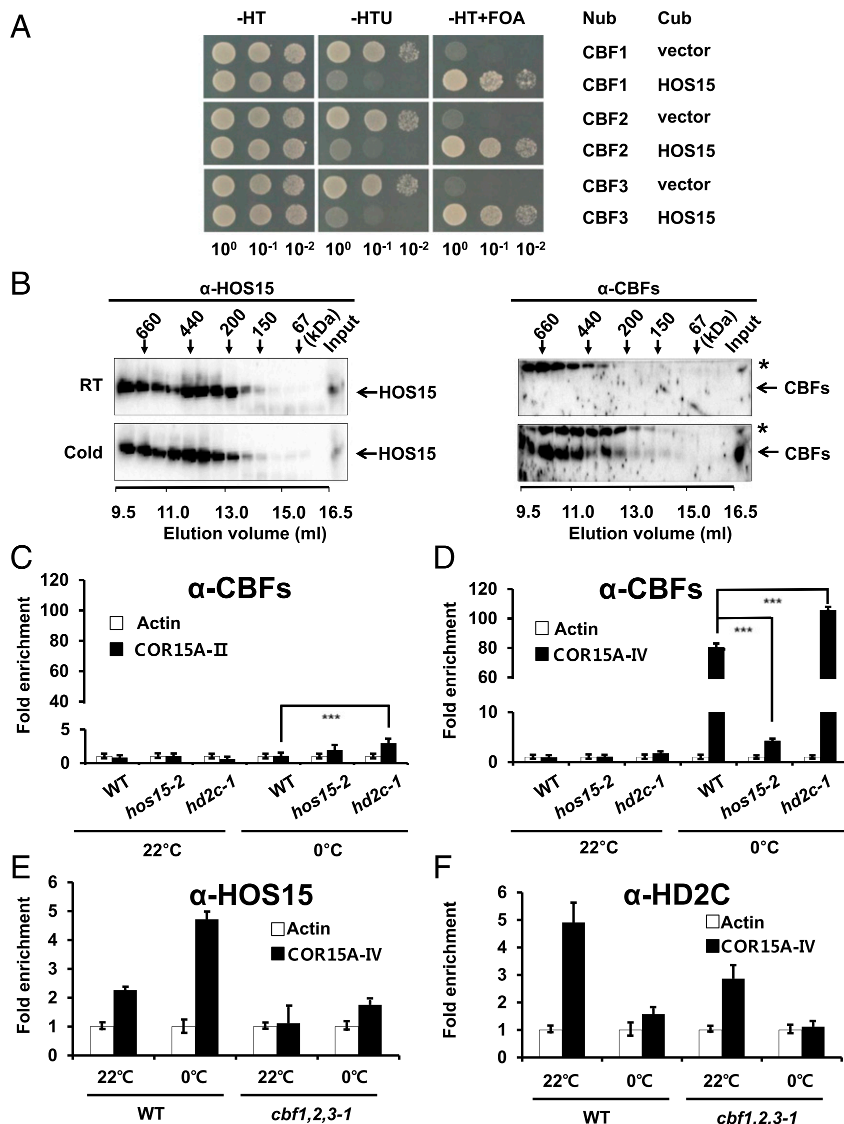

Fig. 6. Cold-induced binding of CBF proteins to *COR15A* promoter is affected by HOS15 and HD2C. (*A*) HOS15 interacts with CBF proteins using yeast split Ub assay. Yeast cells cotransformed with *Nub* (Vector) or *Cub-HOS15* and *CBF1-Nub-RUra3P*, *CBF2-Nub-RUra3P*, or *CBF3-Nub-RUra3P* were spotted on selective media (–HTU and -HTU+5-FOA). Pictures were taken after 4-d incubation at 30 °C. (*B*) HOS15 makes a complex with CBF proteins upon cold treatment. Total protein extracts from wild-type exposed to cold (0 °C) for 24 h were subjected to gel-filtration, using a Superdex 200 10/300 column. Each eluate [0.5 mL 50 mM Tris·Cl (pH7.5) and 100 mM NaCl] was TCA-precipitated, and analyzed by immunoblots with anti-CBFs and anti-HOS15 antibodies. (*C* and *D*) Cold-induced binding of CBF proteins to cold-responsive gene *COR15A* promoter regions, II (*C*), and IV (*D*) is reduced and enhanced in *hos15-2* and *hd2c-1*, respectively. Chromatin from wild-type, *hos15-2*, and *hd2c-1* plants treated with cold (0 °C) for 24 h were immunoprecipitated with anti-CBF antibody (***$P < 0.001$). Similar results were obtained from three independent experiments. (*E* and *F*) Binding of HOS15 and HD2C proteins to cold-responsive gene *COR15A* promoter regions, II, and IV is reduced and in *cbf1/2/3* mutant, respectively. Chromatin from wild-type and *cbf1/2/3* mutant plants treated with cold (0 °C) for 24 h were immunoprecipitated with anti-HOS15 (*E*) or anti-HD2C (*F*) antibodies. Similar results were obtained from three independent experiments.

(Fig. 5). We suggest that CBFs, expressed at basal levels, recruit HOS15 to target *COR* genes and that upon cold signaling HOS15 tags HD2C for degradation, thereby initiating the transition of chromatin to an open state that, in turn, facilitates the accessibility of newly synthesized CBFs. The factors controlling how chromatin remodeling enzymes are targeted to specific loci is an area of intense research (59), and the extension of our model to other gene regulons could explain in mechanistic terms how specific chromatin sites are chosen to be remodeled for transcriptional regulation. Identification of HOS15-interacting transcription factors and other components of the HOS15-containing corepressor and coactivator complexes will help to substantiate our propositions.

TBL1 acts as a bridge between the corepressor and coactivator proteins by dismissal and subsequent degradation of the corepressors N-CoR/SMRT, and for the subsequent recruitment of the coactivator complexes. Our results suggest a possible role of HOS15 as a corepressor/coactivator exchange factor in plants (Fig. 5). Signal-dependent modulation of gene transcription is a key step in stress gene regulation. When overexpressed (i.e., deregulation of the stress-induced gene expression), many stress-responsive genes cause increased stress tolerance at the expense of retarded growth (60, 61). Thus, a proper on–off regulation of the stress gene transcription is necessarily required for the right balance between growth and stress tolerance (47). Our data indicate that, when the cold signal comes, HOS15 interacts with CUL4 and promotes degradation of HD2C in the nucleus. Thus, it appears that HOS15 follows a different mechanism in the corepressor/coactivator exchange process, omitting the dismissal of corepressors that is achieved by TBL1 in animals. These results seem to be related to the rapid recruitment of CBFs to *COR* gene chromatin, as the main coactivator function of HOS15. When cold stress began, CBFs were immediately induced and then bound to *COR* promoters, which increased *COR* gene expression. In this case, the corepressor dismissal step may be omitted because HOS15 rapidly induces degradation of HD2C and recruitment of CBFs to *COR* promoters.

The cold acclimation process allows hardy plants to mount the mechanisms needed for the acquisition of freezing tolerance. CBF-mediated expression of *COR* genes is a key regulatory step of cold acclimation. A dynamic balance between histone acetylation and deacetylation determines the expression of *COR* genes, and thus the cold-response status of the plant. Under ambient temperature, HDACs target nucleosomes surrounding transcription start sites (TSSs) of *CBF* genes and other positive effectors, restricting their expression. Concomitantly, negative effectors are targeted by HATs as to promote their expression. This results in the inhibition of *COR* gene expression under normal ambient temperature. On perception of the low-temperature signal, HATs and HDACs shift roles to target nucleosomes of positive and negative effectors, respectively. Moreover, HATs directly acetylate nucleosomes surrounding the TSSs of *COR* genes. Additional evidence suggests that the HAT GCN5 is capable of clearing nucleosomes at the TSSs of *COR* genes. GCN5 is recruited by the CBF1 transcription factor through the transcriptional adaptor ADA2 to enhance the expression of target *COR* genes (62). The overall effect is an induction of *COR* gene expression at low temperature, leading to increased freezing tolerance. Our data showed that the *hos15* mutant has significantly decreased freezing tolerance after cold acclimation (Fig. 2). These results suggest that although expression of CBFs was increased, the absence of HOS15 could impair CBF recruitment to *COR* gene promoters. Of note, the original *hos15-1* mutant (C24 background) was identified in a forward screen as showing higher expression of the *RD29A:LUC* reporter gene in response to cold, salt, and ABA, although the *hos15-1* mutant was only sensitive to cold (37). Thus, it was concluded that higher expression of *COR* genes in *hos15-1* could result from an increased stress signal arising from the hypersensitivity to cold. Here we show that the *hos15-2* mutant in Col-0 background had lower expression of *COR15A*, *COR47*, and *RD29A* compared with wild-type. Discrepancies could result from the different alleles used or the differential sensitivity of Col-0 and C24 to the cold treatment.

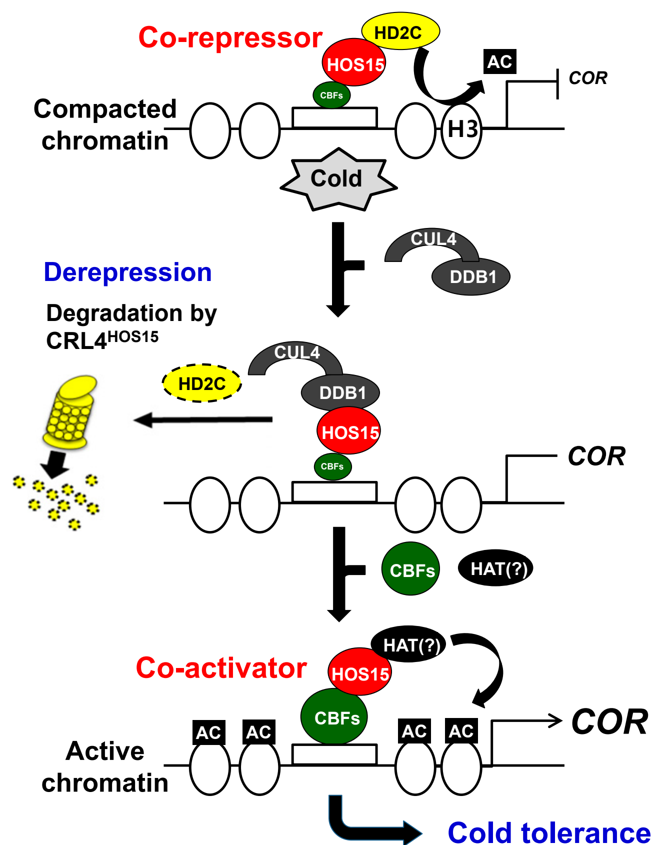

Fig. 7. Model for the HOS15-mediated chromatin remodeling in response to cold stress. In the absence of cold stress, HOS15 forms a complex with HD2C to repress *COR* gene expression by hypoacetylation of *COR* chromatin. Under cold stress conditions, HOS15 recruits CUL4 to form a $CRL4^{HOS15}$ complex, resulting in degradation of HD2C via the Ub–proteasome system. Dissociation of HD2C by $CRL4^{HOS15}$ results in the hyperacetylation of H3 on *COR* chromatin and induces the association of CBF transcription factors to the *COR* promoters via HOS15, thereby increasing *CORs* expression and cold tolerance. The unknown factor (question mark) recruiting HOS15 to *COR* genes under temperate conditions might also be CBFs expressed at basal levels (see *Discussion* for details).

Epigenetic regulation plays important roles in many aspects of abiotic stress processes. Our study suggest that epigenetic regulation in the cold stress responses is an essential part of the *COR* gene expression that is key to cold tolerance. We report here the molecular mechanism of HOS15-mediated chromatin remodeling in response to cold stress. In normal conditions, HOS15 interacts with HD2C and these are associates to CTR/DRE elements in *COR* gene promoter regions. The HOS15–HD2C complex deacetylates *COR* chromatin to repress gene expression. Under cold stress, HOS15 induces ubiquitination and degradation of HD2C, which correlates with increased levels of acetylated histones on the chromatin of *COR* genes, resulting in the promotion of gene transcription in association with the CBF proteins binding to the “open” *COR* gene chromatin for cold tolerance (Fig. 7).

Materials and Methods

Plant Materials and Growth Conditions. *hos15-2* (GK_785B10) and *hd2c-1* (SALK_129799) are in the Colombia (Col-0) background. Mutant *cul4-1* and transgenic lines of *35S::FLAG-DDB1B* and *35S::FLAG-CUL4* are kind gifts from Xing Wang Deng at Peking University, Beijing (33, 63). Genotypes were determined by genomic DNA PCR. All seeds were sterilized with 70% ethanol and 2% bleach (sodium hypochlorite solution, NaOCl) and stratified at 4 °C for 2–3 d. Plants were grown under long-day conditions (16 h light/8 h dark, 80–100 μM $m^{-2}s^{-1}$) at 23 °C.

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Freezing Tolerance and Electrolyte Leakage Measurements. The freezing tolerance assay was performed as previously described (64), with some modifications. Briefly, 3-wk-old seedlings on the soil treated with or without cold acclimation at 4 ± 1 °C for 7 d were moved to the freezing chamber (RUMED, P350) in which temperatures went down every 2 °C for 0.5 h (nonacclimated condition) and maintained for 1 h, and it dropped up to the indicated temperatures. Then plants were covered with ice in the dark for 24 h at 4 ± 1 °C, and transferred under the light at 23 °C. The survival rate was determined in 5 d. For electrolyte leakage measurement, a rosette leaflet from 3-wk-old plants was incubated in 200 μL deionized H_2O in a refrigerated circulator (freezing bath, model AP28R, Poly Science), which temperature was programmed to decrease from 0 °C to −10 °C with 1 °C decrements every 30 min. The sample was then transferred to another tube containing 25-mL deionized H_2O and shaken overnight, and the conductivity of the solution was measured using a 4-Electrode Cell (013005MD; Thermo Scientific Orion). The tubes with the leaflets were then autoclaved, and after cooling to room temperature, conductivities of the solutions were measured again. Percent electrolyte leakage was calculated as the conductivity before autoclaving as a percentage of that after autoclaving.

Yeast Two-Hybrid Interaction Assays. For yeast two-hybrid assays, the bait (pAS2-HOS15, Trp selection) and the prey (pACT2-HD2C or pACT2-DDB1B, Leu selection) fusion constructs were cotransformed into *Saccharomyces cerevisiae* strain PJ69-4A using PEG and heat shock (Clontech protocol). Growth of transformants was observed on synthetic complete medium lacking Trp, Leu, His, and supplemented with 20 mM 3-AT (3-amino-2,3,5-triazole). Plates were photographed after incubation at 30 °C for 5 d. Assays were each performed three times, and each experiment included three biological replicates. For split-ubiquitin yeast two-hybrid assays (65), HOS15, and CBF proteins were cloned into a bait vector, pMet (pMet-GWY-Cub-URA3p-CYC1) and a prey vector, pNuI (pCup-NuI-GWY–CYC1), respectively, and were transformed into *S. cerevisiae* strain JD53 by PEG-mediated heat shock. Interactions were tested by the cell growth on selective medium containing 1 mg/mL 5-FOA (5-Fluoroorotic acid monohydrate; Zymo Research) and deficient uracil medium. Plates photos were taken after 3- to 5-d incubation at 30 °C.

Split Luciferase Complementation Assays. *HOS15* cDNA was cloned into *pCambia1300-cLUC*, and *HD2C* was into *pCambia1300-nLUC* plasmids, respectively (41). *Agrabacterium tumefaciens* strain GV 3101 carrying HOS15-CLuc and HD2C-NLuc (final OD = 0.5 at 600 nm) was infiltrated into tobacco leaves. After 3 d, the abaxial side of the leaves was sprayed with 1 mM luciferin and applied to an EM CCD camera (iXon; Andor Technology). Bioluminescence was detected for 5 min after 5-min quenching in the dark.

Immunoprecipitation for Interactions Between HOS15 and HD2C or CUL4 Complex Proteins. Fusions corresponding to HOS15-FLAG together with HD2C-GFP or DDB1A-HA or DDB1B-HA were transiently expressed in tobacco. Total protein extracts were incubated with protein A agarose fused to anti-FLAG or anti-HOS15 at 4 °C for 1 h. Complexes were separated by SDS/PAGE and the immunoblot was incubated with the appropriate primary antibody [anti-GFP (1:5,000; Albcam); anti-HA (1:2,000; Roche); and anti-HOS15 (66) (1:200)] overnight at 4 °C. Antigen protein was detected by chemiluminescence using an ECL-detecting reagent (Thermo Scientific).

BiFC Assay. pDEST-^{GW}VYNE-HOS15 and pDEST-^{GW}VYCE-HD2C constructs were transformed into *Arabidopsis* protoplasts using PEG transfection (67) and YFP fluoroscence was detected in 12-h cold treatment (4 °C) under a confocal laser scanning microscope (Olympus FV1000). Nuclei were stained by DAPI (6-diamidino-2-phenylindole, 1 μg mL^{-1}; Sigma). YFP filter set up: excitation 514 nm and captured at 560–610 nm.

RNA Extraction and Expression Analysis. Total RNA from 14-d-old plants was extracted using the RNeasy plant Mini kit (Qiagen) and reverse-transcribed using SuperScriptII reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix kit (Bio-Rad SYBR Green Supermix) with the CFX96 or CFX384 real-time PCR detection system (Bio-Rad). The relative expression levels were calculated using the comparative cycle threshold method. The primers used for qRT-PCR are listed in *SI Appendix*, Table S2.

Nuclei Isolation and Western Blot Analysis. Nuclei were extracted from 14-d-old seedlings by using Honda's buffer (2.5% Ficoll 400, 5% dextran T-40, 0.4 M sucrose, 25 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM mercaptoethenol, 100 mg/mL of phenylmethylsulfonyl fluoride, 0.5 mg/mL of antipain, and 0.5 mg/mL of leupeptin) (68). Nuclear proteins were separated by SDS/PAGE. Immunoblots were performed using appropriate antibodies, and antigen proteins were visualized by chemiluminescence using ECL-detecting reagent (Thermo Scientific).

Ubiquitination Assays. For in vivo detection of ubiquitinated HD2C-GFP in *GFP-HD2C* and *GFP-HD2C/hos15-2* plants, 7-d-old seedlings pretreated with 50 μM proteasome inhibitor MG132 were incubated at 4 °C for 12 h. Total protein extracts were incubated with p62 resins (69) and were separated by SDS/PAGE. The immunoblots were carried out with anti-Ub (1:1,000; Boston Biochem) and anti-GFP (1:1,000; Miltenyi Biotec).

ChIP Assay. For ChIP assays, 0.5 g of 2-wk-old plants treated with cold (0 °C) for 24 h were treated with 1% formaldehyde for 15 min under vacuum. Glycine was added to a final concentration of 0.1 M, and incubation was continued for an additional 5 min. Plants were then washed with H_2O and ground in liquid N_2. Approximately 0.3 g of the ground sample was resuspended in 1 mL nuclei lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mM Na butyrate, 1 μg/mL aprotinin, 1 μg/mL pepstatin A]. DNA was sheared by sonication (Bioruptor) to ∼500- to 1,000-bp fragments. After centrifugation (10 min at 16,000 × *g*), the supernatants were precleared with 60 μL salmon sperm (SS) DNA/Protein A agarose for 60 min at 4 °C. After 2 min of centrifugation at 16,000 × *g*, the supernatant was transferred to a siliconized tube, and 10 μL of the appropriate antibody was added. Antibodies used were anti-AcH3 (Millipore), antiacetylated H3K9/K14 (Upstate Biotechnology), anti-HOS15 (66), anti-CBFs (70), and anti-HD2C (Agrisera). After incubation overnight with rotation at 4 °C, 60 μL SS DNA/Protein A agarose was added and incubation continued for 2 h. The agarose beads were then washed with 1 mL of each of the following: two times lysis buffer, one time LNDET buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8)], and three times TE buffer [10 mM Tris·HCl (pH 8), 1 mM EDTA]. The immunocomplexes were eluted from the beads with 300 μL elution buffer (1% SDS, 0.1 M $NaHCO_3$). A total of 12 μL 5 M NaCl was then added to each tube, and cross-links were reversed by incubation at 65 °C for 6 h. Residual protein was degraded by the addition of 20 μg Protease K [in 10 mM EDTA and 40 mM Tris (pH 8)] at 45 °C for 1 h, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Precipitated DNA was dissolved in 50 μL TE and 2 μL was used for PCR. Quantitative PCR was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP experiments.

Size-Exclusion Chromatography. Size-exclusion chromatography (SEC) was performed by using an ÄKTA fast-performance liquid chromatography (FPLC) system with prepacked Superdex 200 10/300 GL column (GE Healthcare). The total proteins extracted from *Arabidopsis* wild-type or Flag-CUL4 overexpressing plants with extraction buffer [100 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA] and protease inhibitors (1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL pepstatin, 5 μg/mL antipain, 5 μg/mL chymostatin, 2 mM Na_2VO_3, 2 mM NaF and 50 μM MG132) were loaded onto columns and eluted with elution buffer [50 mM Tris·HCl (pH 8.0), 100 mM NaCl and 0.02% sodium azide] in a flow-rate of 0.5 mL/min at room temperature. The eluted proteins were monitored at OD_{280}. After SEC, each protein fractions (500 μL) was precipitated by mixing with 12.5% trichloroacetic acid (TCA). Precipitated protein pellets were dissolved in urea/SDS buffer and separated in 6% SDS-gel.

ACKNOWLEDGMENTS. We thank Dr. Zhizhong Gong and Dr. Shuhua Yang for their valuable discussion of our experiments; and Dr. Ming Luo at South China Botanical Garden, Chinese Academy of Sciences, for providing *hd2c-3* seed stocks. This work was supported by grants from the National Research Foundation of Korea, funded by the Korean Government (MSIP 2016R1A2A1A05004931 and Global Research Laboratory 2017K1A1A2013146); and Next-Generation BioGreen 21 Program SSAC Grants PJ01318201 (to D.-J.Y.), PJ01318205 (to J.M.P.), and PJ01327301 (to W.-Y.K.), Rural Development Administration, Republic of Korea. Work in the V.R. laboratory was funded by Agencia Estatal de Investigación of Ministerio de Economía, Industria y Competitividad, with support from the European Regional Development Fund (BIO2016-80551-R).

1. Jeon J, Kim J (2013) Cold stress signaling networks in *Arabidopsis*. *J Plant Biol* 56: 69–76.
2. Wensing M, Grol R (1998) What can patients do to improve health care? *Health Expect* 1:37–49.
3. Chinnusamy V, Zhu J, Zhu J-K (2006) Gene regulation during cold acclimation in plants. *Physiol Plant* 126:52–61.
4. Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:571–599.
5. Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: An overview. *Arch Biochem Biophys* 444:139–158.
6. Gong Z, et al. (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc Natl Acad Sci USA* 99: 11507–11512.
7. Hsieh T-H, et al. (2002) Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol* 129:1086–1094.
8. Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* 8:489–503.
9. Lang V, Mantyla E, Welin B, Sundberg B, Palva ET (1994) Alterations in water status, endogenous abscisic acid content, and expression of rab18 gene during the development of freezing tolerance in *Arabidopsis thaliana*. *Plant Physiol* 104:1341–1349.
10. Gilmour SJ, Fowler SG, Thomashow MF (2004) *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol Biol* 54:767–781.
11. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417.
12. Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′-region of *Arabidopsis* thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol Biol* 24:701–713.
13. Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* 6:251–264.
14. Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol* 124:1854–1865.
15. Jia Y, et al. (2016) The cbfs triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in *Arabidopsis*. *New Phytol* 212: 345–353.
16. Zhao C, et al. (2016) Mutational evidence for the critical role of CBF transcription factors in cold acclimation in *Arabidopsis*. *Plant Physiol* 171:2744–2759.
17. Kim J-M, Sasaki T, Ueda M, Sako K, Seki M (2015) Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. *Front Plant Sci* 6:114.
18. Yuan L, Liu X, Luo M, Yang S, Wu K (2013) Involvement of histone modifications in plant abiotic stress responses. *J Integr Plant Biol* 55:892–901.
19. Chinnusamy V, Zhu J, Zhu J-K (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci* 12:444–451.
20. Luo M, et al. (2012) Chromatin modifications and remodeling in plant abiotic stress responses. *Biochim Biophys Acta* 1819:129–136.
21. Moraga F, Aquea F (2015) Composition of the SAGA complex in plants and its role in controlling gene expression in response to abiotic stresses. *Front Plant Sci* 6:865.
22. Pandey R, et al. (2002) Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res* 30:5036–5055.
23. Shahbazian MD, Grunstein M (2007) Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* 76:75–100.
24. Näär AM, Lemon BD, Tjian R (2001) Transcriptional coactivator complexes. *Annu Rev Biochem* 70:475–501.
25. Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14:121–141.
26. Jepsen K, Rosenfeld MG (2002) Biological roles and mechanistic actions of corepressor complexes. *J Cell Sci* 115:689–698.
27. Strahl BD, Allis CD (2000) The language of covalent histone modifications. *Nature* 403: 41–45.
28. Freiman RN, Tjian R (2003) Regulating the regulators: Lysine modifications make their mark. *Cell* 112:11–17.
29. Schwartz DC, Hochstrasser M (2003) A superfamily of protein tags: Ubiquitin, SUMO and related modifiers. *Trends Biochem Sci* 28:321–328.
30. Hotton SK, Callis J (2008) Regulation of cullin RING ligases. *Annu Rev Plant Biol* 59: 467–489.
31. He YJ, McCall CM, Hu J, Zeng Y, Xiong Y (2006) DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases. *Genes Dev* 20:2949–2954.
32. Higa LA, et al. (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol* 8:1277–1283.
33. Lee J-H, et al. (2008) Characterization of *Arabidopsis* and rice DWD proteins and their roles as substrate receptors for CUL4-RING E3 ubiquitin ligases. *Plant Cell* 20:152–167.
34. Pintard L, Willems A, Peter M (2004) Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family. *EMBO J* 23:1681–1687.
35. Lee J-H, et al. (2010) DWA1 and DWA2, two *Arabidopsis* DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. *Plant Cell* 22:1716–1732.
36. Lee J-H, Terzaghi W, Deng XW (2011) DWA3, an *Arabidopsis* DWD protein, acts as a negative regulator in ABA signal transduction. *Plant Sci* 180:352–357.
37. Zhu J, et al. (2008) Involvement of *Arabidopsis* HOS15 in histone deacetylation and cold tolerance. *Proc Natl Acad Sci USA* 105:4945–4950.
38. Kim J, Harter K, Theologis A (1997) Protein-protein interactions among the Aux/IAA proteins. *Proc Natl Acad Sci USA* 94:11786–11791.
39. Colville A, et al. (2011) Role of HD2 genes in seed germination and early seedling growth in *Arabidopsis*. *Plant Cell Rep* 30:1969–1979.
40. Sridha S, Wu K (2006) Identification of AtHD2C as a novel regulator of abscisic acid responses in *Arabidopsis*. *Plant J* 46:124–133.
41. Chen H, et al. (2008) Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiol* 146:368–376.
42. Kato N, Jones J (2010) The split luciferase complementation assay. *Plant Developmental Biology: Methods and Protocols*, eds Hennig L, Köhler C (Humana, Totowa, NJ), pp 359–376.
43. Buszewicz D, et al. (2016) HD2C histone deacetylase and a SWI/SNF chromatin remodelling complex interact and both are involved in mediating the heat stress response in *Arabidopsis*. *Plant Cell Environ* 39:2108–2122.
44. Luo M, et al. (2012) HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. *J Exp Bot* 63:3297–3306.
45. Chinnusamy V, et al. (2003) ICE1: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 17:1043–1054.
46. Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* 280:104–106.
47. Gilmour SJ, et al. (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J* 16:433–442.
48. Jin J, Arias EE, Chen J, Harper JW, Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* 23:709–721.
49. Luo M, Wang Y-Y, Liu X, Yang S, Wu K (2012) HD2 proteins interact with RPD3-type histone deacetylases. *Plant Signal Behav* 7:608–610.
50. Perissi V, et al. (2008) TBL1 and TBLR1 phosphorylation on regulated gene promoters overcomes dual CtBP and NCoR/SMRT transcriptional repression checkpoints. *Mol Cell* 29:755–766.
51. Rosenfeld MG, Lunyak VV, Glass CK (2006) Sensors and signals: A coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 20:1405–1428.
52. Chen X, et al. (2016) POWERDRESS interacts with HISTONE DEACETYLASE 9 to promote aging in *Arabidopsis*. *eLife* 5:e17214.
53. Kim YJ, et al. (2016) POWERDRESS and HDA9 interact and promote histone H3 deacetylation at specific genomic sites in *Arabidopsis*. *Proc Natl Acad Sci USA* 113: 14858–14863.
54. Privalsky ML (2004) The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu Rev Physiol* 66:315–360.
55. Miura K, Furumoto T (2013) Cold signaling and cold response in plants. *Int J Mol Sci* 14:5312–5337.
56. Choi H-K, et al. (2011) Reversible SUMOylation of TBL1-TBLR1 regulates β-catenin-mediated Wnt signaling. *Mol Cell* 43:203–216.
57. Bosu DR, Kipreos ET (2008) Cullin-RING ubiquitin ligases: Global regulation and activation cycles. *Cell Div* 3:7.
58. Dong MA, Farré EM, Thomashow MF (2011) Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in *Arabidopsis*. *Proc Natl Acad Sci USA* 108:7241–7246.
59. Gibney ER, Nolan CM (2010) Epigenetics and gene expression. *Heredity (Edinb)* 105: 4–13.
60. Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep* 25:1263–1274.
61. Chinnusamy V, Zhu J-K (2009) Epigenetic regulation of stress responses in plants. *Curr Opin Plant Biol* 12:133–139.
62. Mao Y, Pavangadkar KA, Thomashow MF, Triezenberg SJ (2006) Physical and functional interactions of *Arabidopsis* ADA2 transcriptional coactivator proteins with the acetyltransferase GCN5 and with the cold-induced transcription factor CBF1. *Biochim Biophys Acta* 1759:69–79.
63. Chen H, et al. (2006) *Arabidopsis* CULLIN4 forms an E3 ubiquitin ligase with RBX1 and the CDD complex in mediating light control of development. *Plant Cell* 18:1991–2004.
64. Ji H, et al. (2015) The *Arabidopsis* RCC1 family protein TCF1 regulates freezing tolerance and cold acclimation through modulating lignin biosynthesis. *PLoS Genet* 11: e1005471.
65. Stagljar I, Korostensky C, Johnsson N, te Heesen S (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc Natl Acad Sci USA* 95:5187–5192.
66. Lee S, et al. (2012) Monoclonal antibodies against recombinant AtHOS15. *Hybridoma (Larchmt)* 31:118–124.
67. Gehl C, Waadt R, Kudla J, Mendel R-R, Hänsch R (2009) New GATEWAY vectors for high throughput analyses of protein-protein interactions by bimolecular fluorescence complementation. *Mol Plant* 2:1051–1058.
68. Folta KM, Kaufman LS (2006) Isolation of *Arabidopsis* nuclei and measurement of gene transcription rates using nuclear run-on assays. *Nat Protoc* 1:3094–3100.
69. Irigoyen ML, et al. (2014) Targeted degradation of abscisic acid receptors is mediated by the ubiquitin ligase substrate adaptor DDA1 in Arabidopsis. *Plant Cell* 26:712–728.
70. Cho S, et al. (2017) Accession-dependent *CBF* gene deletion by CRISPR/Cas system in *Arabidopsis*. *Front Plant Sci* 8:1910.

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