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

Junghoon Park, Chae Jin Lim, Mingzhe Shen, Hee Jin Park, Joon-Yung Cha, Iniesta, Vicente Rubio, Tesfaye Mengiste, Jian-Kang Zhu, Ray A. Bressan, Yeol Lee, Byeong-ha Lee, Jing Bo Jin, Jose M. Parodo, Woe-Yeon Kim, and Dae-Jin Yun

Department of Biomedical Science and Engineering, Konkuk University, 05029 Seoul, South Korea; Division of Applied Life Science, BK21 plus Program, Plant Molecular Biology and Biotechnology Research Center, Institute of Agriculture and Life Science, Gyeongsang National University, 52828 Jinju, Republic of Korea; Institute of Glocal Disease Control, Konkuk University, 05029 Seoul, Republic of Korea; Plant Molecular Genetics Department, Centro Nacional de Biotecnologia-Consejo Superior de Investigaciones Científicas, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain; Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907; Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907; Department of Life Science, Sogang University, 04107 Seoul, South Korea; Institute of Botany, Chinese Academy of Sciences, 100093 Beijing, China; and Institute of Plant Biochemistry and Photosynthesis, Consejo Superior de Investigaciones Científicas, 41092 Seville, Spain

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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

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.

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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.

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 andABA-independent expression of COLD RESPONSIVE (COR)/RESPONSIVE TO DEISCICATION (RD)/LOW-TEMPERATURE–INDUCED (LTI)/KIN (stress-induced) genes through association to CRY/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 regulators 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.

Epigenetic mechanisms, including histone modifications, chromatin remodeling, and DNA methylation, are known to play a critical role in gene expression (28). The activity of transcription factors is often regulated at the chromatin level, which is considered to be the starting point for gene transcription (29). However, the role of histone modifications in cold-responsive genes remains largely unexplored.

In Arabidopsis, cold-responsive genes are regulated by the CBF/DREB (CORycin DEHYDRATION RESPONSIVE ELEMENT BINDING FACTORS) transcription factors, which control the expression of genes involved in stress response and tolerance (30). However, the molecular mechanisms underlying the regulation of cold-responsive gene expression remain largely unknown. Here, we report that the HOS15 protein, a WD40-repeat protein, interacts with histone deacetylase 2C (HD2C) and regulates the acetylation of histones H3 and H4 at the chromatin level. Our results demonstrate that HOS15 promotes the degradation of HD2C in a cold-dependent manner, leading to the derepression of cold-responsive genes. We propose a model in which HOS15 acts as a substrate receptor for HD2C degradation, thereby facilitating the recruitment of CBF transcription factors to the chromatin of cold-responsive genes. This epigenetic switch enables plants to adapt to cold stress and survive in adverse environments.
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 CUL14 (CUL4) assembles a small RING-box domain protein (RBX1) on its C terminus and the DNA BINDING PROTEIN 1 (DBB1) on its N terminus to interact with substrate receptors, namely DCAFs (CUL- and DBB1-associated factors), that recognize their corresponding targets for ubiquitination. The DCAFs usually possess the DBB1-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. Thus, 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. 1A and SI Appendix, Table S1). As we were more interested in how HOS15 is involved in epigenetic regulation, the interaction of HOS15 and HD2C (SI Appendix, Table S1) was confirmed using chromoprecipitation (co-IP) (Fig. 1B) (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. 1B). 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 luminescence signals (Fig. 1C and SI Appendix, Fig. S1), revealing physical interaction between HOS15 and HD2C. Consistent with the known localization of HOS15 in nuclei (37), YFP<sup>HOS15</sup> expressed in Arabidopsis protoplasts interacted with YFP<sup>HD2C</sup> in the nucleus as shown by biomolecular fluorescence complementation (BiFC) assays (Fig. 1D).

HOS15 and HD2C Are Involved in Freezing Stress Responses. As the hos15-7 allele is in the C24 ecotype background (37) and hos15-2 (GK_758510) is in Col-0 background, the complementation lines /<sup>hos15</sup>5pro::/<sup>hos15</sup>-HA and transgenic plants expressing /<sup>hos15</sup>-FLAG were generated in hos15-2 (SI Appendix, Fig. S2A–E). We also obtained two mutant alleles of HD2C, <sup>hd2c</sup>-1 (SALK_129799) and <sup>hd2c</sup>-3 (SALK_039784), and transgenic plants overexpressing HD2C-GFP (HDD2Cox) (40, 43, 44) (SI Appendix, Fig. S3 A–E). The hos15-2 loss-of-function mutant exhibited cold sensitive phenotypes, with and
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 G–I). 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. 2D and SI Appendix, Fig. S4A). 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. 2D). 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. 2D). 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 were 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 HD2Cox (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.
sustaining 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. 3A). 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. 3B and C). In addition, HOS15 associated with CUL4, the scaffold component in CRL4 complexes (Fig. 3D), and such interaction was enhanced upon cold treatment in Arabidopsis (Fig. 3E), suggesting that HOS15 assembles into CRL4 E3 Ub ligase complexes (henceforth CRL4HO5).

Because HOS15 interacted with CRL4 components, we tested whether HD2C also associates to CUL4 and DDB1B using co-IP (SI Appendix, Fig. S6D). Total protein extracts from wild-type, ho2c-1, FLAG-CUL4 overexpressor (FLAG-CUL4), and FLAG-DDB1B/dbb1a (FLAG-DDB1B) plants were immunoprecipitated with anti-H2C antibody, and both DDB1B and CUL4 fusions were found to coprecipitate with HD2C, suggesting that HD2C also associates to CRL4HOS5 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 pathway (33, 35). According to this notion, we tested whether HD2C serves as a substrate of the CRL4HOS5 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. 4A and B and SI Appendix, Fig. S5). Disappearance of GFP fluorescence from HD2C-GFP overexpressing plants was also promoted by cold treatment (Fig. 4C). Furthermore, treatment with the proteasome inhibitor MG132 during cold treatment abolished the cold-induced decrease in the steady-state levels of HD2C protein (Fig. 4D), 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. 4D and SI Appendix, Fig. S6E), and CUL4 defective plants were sensitive to freezing (SI Appendix, Fig. S6A–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. 4E). The p62 affinity-purified samples showed extensive ubiquitination, as detected in immunoblots with antiubiquitin antibody (Fig. 4E, 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 polyubiquitin chains, that HOS15 is required for cold-induced polyubiquitination of HD2C (Fig. 4E, Right), and imply that cold sensitivity of hos15-2 results from the failure of HOS15-mediated HD2C degradation. Indeed, hos15-2 ho2c-1 double-mutant plants suppressed the cold-sensitive phenotypes of hos15-2 (SI Appendix, Fig. S7A–C). Furthermore, the

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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 DDB1 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 DDB1 or CUL4. Total proteins from 12-d-old jss: Flag-DDB1B/Flag-CUL4 and jss::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.
reduced expression of COR genes in hos15-2 was also suppressed by hd2c-1 (SI Appendix, Fig. S7D). 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 to 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. 5B). 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. 5H) (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. 5H) (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. 5G) (22 °C). However, the increased binding of HOS15 to COR15A chromatin upon cold stress was not observed in the hos15-2 mutant (Fig. 5G) (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 CBFs isoforms (Figs. 6A and SI Appendix, Fig. S9A and B). In contrast, HD2C failed to interact with all CBFs (SI Appendix, Fig. S9C) 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. 6B). Thus, we examined whether HOS15 and HD2C affected the binding of CBF proteins to CBFs 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
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 COR154 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 co-repressor complex recruits HDAC3 to gene promoter regions (51). The Arabidopsis N-CoR homolog PWR (powerless) 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.

![Image](image-url)
(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.

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-DDB1 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⁻²s⁻¹) at 23 °C.
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 MgCl₂, 10 mM mercaptoethanol, 100 mM mg/l of phenylmethylsulfonflouride, 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/ho1-2 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; Milteny Biotech).

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 H₂O and ground in liquid N₂. Approximately 0.3 g of the ground sample was resuspended in 1 ml nuclease 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 peptatin A]. DNA was sheared by sonication (Bioruptor) to ~500–1,000-bp fragments. After centrifugation (10 min at 16,000 g), the supernatants were pre cleared 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 × 10 × g, the supernatant was transferred to a sonication tube, and 10 μl of the appropriate antibody was added. Antibodies used were anti-ACh5 (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 LNDT 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 NaHCO₃). 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 NaVO₃, 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 OD280. 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.

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