Journal of Plant Interactions

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjpi20

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To cite this article: Jae Ho Kim, Irfan Ullah Khan, Moon Seok Kim & Yong Weon Seo (2021) Functional characterization of wheat histone H2B monoubiquitination enzyme TaHUB2 in response to vernalization in Keumkang (*Triticum aestivum* L.), Journal of Plant Interactions, 16:1, 93-103, DOI: 10.1080/17429145.2021.1903104

To link to this article: https://doi.org/10.1080/17429145.2021.1903104

Published online: 24 Mar 2021.

JOURNAL OF PLANT INTERACTIONS
2021, VOL. 16, NO. 1, 93–103
https://doi.org/10.1080/17429145.2021.1903104

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PLANT-ENVIRONMENT INTERACTIONS

Functional characterization of wheat histone H2B monoubiquitination enzyme TaHUB2 in response to vernalization in Keumkang (*Triticum aestivum* L.)

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ABSTRACT

Histone monoubiquitination has an important role in the regulation of DNA replication, repair, and transcription in plants. Here, we report the molecular function of wheat histone H2B monoubiquitination enzyme 2 (TaHUB2). The full length of TaHUB2 contains a Really Interesting New Gene (RING) domain and its encoded protein was localized in both nucleus and cytoplasm. We also find TaHUB2 directly interacts with histone H2B in yeast and tobacco. The transcription level of TaHUB2 was decreased as the increased vernalization periods until 50 d. The TaHUB2 exhibited ubiquitination activities and were rapidly degraded in the cell-free extracts with apparently 3–6 h after vernalization compared with non-vernalization. Moreover, histone H2B was mono-ubiquitinated by TaHUB2 and ubiquitylated histone H2B (H2Bub1) level was decreased after vernalization conditions in wheat. These results provide novel information for understanding the molecular characterization of wheat RING-type E3 ligase and their possible roles.

ARTICLE HISTORY
Received 29 July 2020
Accepted 10 March 2021

KEYWORDS
Wheat; vernalization; histone H2B; RING-type E3 ligase; monoubiquitination; TaHUB2

Introduction

Vernalization is critical for transitioning from vegetative to reproductive organ development in overwintering crops such as winter wheat. Physiological studies have revealed that many plant species have different vernalization properties (Michaels and Amasino 2000; Gendall et al. 2001). Vernalization usually occurs during winter when the temperatures are between 0°C to 10°C (Takahashi and Yasuda 1971). Without vernalization, many cereal and Brassicaceae groups only produce vegetative organs, such as leaves, which leads to a loss of floret fertility and grain yield (Flood and Halloran 1984; Motoki et al. 2019). Moreover, vernalization accelerates spikelet development and rapid floret abortion (Trevaskis 2010; Deng et al. 2015). The *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) genes, which belong to the vernalization pathway, are related to flowering and have been reported in *Arabidopsis thaliana* (Koornneef et al. 1994; Lee et al. 1994). *FRI* positively regulates the transcript level of *FLC*, which encodes the MAD box domain and is negatively regulated by vernalization (Michaels and Amasino 1999; Gendall et al. 2001). Conversely, *FLC* negatively regulates the expression of downstream flowering-related genes such as *FT* and *SUPRESSOR OF OVEREXPRESSION IF CO1* (*SOC1*). *FLC* is the central repressor of flowering time in the vernalization pathway in *Arabidopsis*, whereas, in wheat and other temperate cereals, an *Arabidopsis FLC* homologous gene has not been clearly identified (Michaels and Amasino 1999; Yan et al. 2004). Diploid wheat has two tandemly duplicated genes named *ZCCT1* and *ZCCT2* in the VRN2 locus, encoding proteins with 76% similarity, each with a putative zinc finger and CCT domain. Moreover, the wheat *ZCCT1* (*VRN2*) gene, which belongs to a different transcription family but has a floral repressor function similar to that of the MADS-BOX gene *FLC*, is downregulated in response to vernalization (Yan et al. 2004). Previous studies suggested that the vernalization pathways in *Arabidopsis* and temperate grasses are independently evolved (Yan et al. 2004; Dubcovsky et al. 2006).

Ubiquitin-mediated protein modifications have been extensively reported to play a role in plant growth, development, flowering time, and biotic and abiotic stress tolerance (Zhu et al. 2008; Lim et al. 2013; AN et al. 2017; Hong et al. 2020). Furthermore, ubiquitination can be classified into two major types, monoubiquitination and polyubiquitination, which are determined by either a single ubiquitin or polymerized ubiquitin chain attached to the substrate, respectively (Ramanathan and Ye 2012). Generally, ubiquitination requires three types of key enzymes: an activating enzyme (E1), conjugating enzyme (E2), and ubiquitin ligase (E3) (Stone et al. 2005). Polyubiquitination recognizes and degrades the substrate using the 26s proteasome in an ATP-dependent manner (Smalle and Vierstra 2004). Alternatively, monoubiquitination does not lead to the degradation of the substrate through the 26s proteasome; instead, a single moiety can function in biological regulation, and, together with other types of post-translational modifications such as acetylation, methylation, phosphorylation, and sumoylation, it can regulate transcription, protein trafficking, and DNA repair (Zhu et al. 2005; Braun and Madhani 2012; Feng and Shen 2014).

Histone ubiquitination along with other types of genetic and epigenetic regulation can modulate DNA methylation (Zhang 2003; Shilatifard 2006; Feng et al. 2007; Shen and Casaccia-Bonnefil 2008), the changes in histone variants (H2A, H2B, H3, and H4), and the post-translational modifications of histones (Zhang 2003; Shilatifard 2006; Shen and Casaccia-Bonnefil 2008; Bowman and Poirier 2015). Monoubiquitinated histone H2B (H2Bub1) has been previously reported in yeast and humans; in yeast, the Bre1 E3 ligase

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Supplemental data for this article can be accessed at https://doi.org/10.1080/17429145.2021.1903104

monoubiquitinates H2B through interactions with Rad6 (E2), targeting H2B at the K123 site (Turco et al. 2015). Another study has shown that the RNF20/40 complex ubiquitinates human H2B and then stimulates HOX gene transcription (Zhu et al. 2005). The ubiquitination site of H2B is highly conserved at the K (lysine) residue in yeast (H2BK123), humans (H2BK120), and *Arabidopsis* (H2BK143) (Feng and Shen 2014). Integrative marking analysis of the chromatin region revealed that, in *Arabidopsis*, H2Bub1 is associated with histone H3 trimethylated on K4 (H3K4me3) and K36 (H3K36me3) (Roudier et al. 2011). In the early events of photomorphogenesis, gene upregulation was due to an increase in the H2Bub level in *Arabidopsis* (Bourbousse et al. 2012). It has been recently reported that histone H2B is monoubiquitinated by the enzymes HISTONE MONOBUIQUITINATION1 (HUB1) and HUB2 in *Arabidopsis* (Liu et al. 2007; Cao et al. 2008; Chen et al. 2019). AtHUB1 and AtHUB2, the two E3 ligases, were recently identified as reproductive stage repressors that regulate FLC/MAFs through histone methylation. During the floral transition from the vegetative to reproductive stages, the *hub1* and *hub2* double mutant lines showed an early flowering phenotype by inhibiting H2Bub1 (Cao et al. 2008). However, not much is known about the role of HUB2 during different vernalization periods in wheat development systems.

In recent years, huge breakthroughs have been made in recognizing the function of post-translational modifications (PTMs) such as histone H3 methylation in the control of plant gene expression. The most well understood example of plant histone variants and PTMs is the *Arabidopsis* regulation of the *FLC* gene, which mediates the transition from vegetative to flowering growth. The *FLC* gene is activated during the vegetative growth process and serves to inhibit floral transition by reducing the expression of flowering promoting genes, which indicates that the *FLC* gene must be suppressed for flowering to occur (Deal and Henikoff 2011). During vegetative development, the expression of *FLC* is stimulated by the deposition of H3K4me3 by the *Arabidopsis* Paf1 complex and other trxG proteins (He et al. 2004; Tamada et al. 2009) and by the integration of H2A.Z by the SWR1-like complex (Deal et al. 2007; March-Díaz et al. 2007; Lázaro et al. 2008; March-Díaz et al. 2008). The loss of the Paf1 complex, the SWR1-like complex, the trxG proteins, or H2A.Z results in decreased *FLC* expression, which promotes premature flowering (Deal and Henikoff 2011). In addition to these, further modifications such as H2B ubiquitination, H3 acetylation, and H3K36 methylation are also involved in the maintenance of *FLC* expression and the suppression of flowering (He 2009).

In the present study, we characterized wheat *TaHUB2*, which encodes the Really Interesting New Gene (RING) finger protein and has sequence similarity with *Arabidopsis AtHUB2*. *TaHUB2* physically interacts with H2B, UBC1, and UBC2 in yeast two-hybrid, bimolecular fluorescence complementation (BiFC) and in vitro pull-down assays and possesses monoubiquitination activity. Furthermore, we found that the transcription level of *TaHUB2* decreased under an extended period of vernalization in a wheat cultivar (cv. *Keumkang*). Moreover, the levels of H2Bub1, H3K4me3, and H3K36me3 reduced significantly under extended vernalization. This indicates that the vernalization periods mediate differential histone methylation patterns, which might be involved in the rapid heading time compared with that under non-vernalized conditions.

Materials and methods

Plant material and growth conditions

The seeds of a wheat variety (*Triticum aestivum* L, cv. *Keumkang*) were vernalized in a cold room at 4°C in the dark for 0, 10, 20, 30, or 50 days. At the end of vernalization, the plants were transferred to soil pots in a greenhouse at 25°C under 16-h/8-h light/dark photoperiod conditions. The heading dates of three plants from each experimental unit (0, 10, 20, 30, and 50 days) were recorded. Samples were harvested at different vernalization times (0, 10, 20, 30, and 50 days) at four leaf stages (Zadoks growth scale 14–15) and stored at −80°C until analysis.

Gene cloning and vector construction

To isolate the full sequence of TaHUB2, primer sets were designed based on sequences downloaded from wheat IWGSC Reference Sequence v1.0 and the Ensemble database (http://plants.ensembl.org/Multi/Tools/Blast). We selected TraesCS3A02G467300 E3 ligase genes for further analysis (Supplementary Table S1). The open reading frames (ORFs) of the *TaHUB2* gene were amplified from wheat cDNA and then inserted into the pCR8/GW/TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). The ORFs of *TaHUB2* were inserted into pK7FWG2 to obtain TaHUB2-GFP constructs using the GATEWAY system. The ORFs of *TaHUB2* were cloned into pMAL-c5x vectors to generate maltose-binding protein (MBP)-TaHUB2. The ORFs of *TaHUB2* were also cloned into pGADT7 (prey) vectors to generate pGADT7-TaHUB2. The primers used in this study are listed in Supplementary Table S1.

Gene expression studies

To identify the expression patterns of the genes, we carried out a qRT-PCR as previously described (Yoon et al. 2019). Total RNA was extracted using TRIzol® reagent (Invitrogen). First-strand cDNA was synthesized from the total RNA (2 μg) using a Power cDNA Synthesis kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) following the manufacturer's instructions. Real-time qPCR was performed using SYBR Green (Applied Biological Materials Inc., Richmond, British Columbia, Canada) and monitored using a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The amplification protocol was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 1 min at 95°C, 30 s at 58°C, 1 min at 72°C, and a final elongation step at 72°C for 5 min. The qPCR results were calculated using the delta-delta CT method (Livak and Schmittgen 2001). Three biological replicates were performed for all qRT-PCR experiments.

Subcellular localization

To identify the subcellular localization of TaHUB2, each gene was cloned into the pK7FWG2 (C-terminal fragment of GFP) vector using LR clonase (Thermo Fisher Scientific, Waltham, MA, USA). The plasmid 35S::GFP- TaHUB2 was introduced into *Agrobacterium tumefaciens* GV3101 using the freeze–thaw method. Agrobacteria carrying 35S::GFP-TaHUB2 were grown in LB medium until the optical density

of the cultures reached 1.0 at 600 nm. The *Agrobacterium* cells were pelleted at 4000 rpm for 10 min at room temperature and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 200 mM acetosyringone, pH 5.6). Then, tobacco leaves were injected with the *Agrobacterium* solutions using needleless syringes. All constructs were then introduced into *Agrobacterium* strain GV3101 and infiltrated into tobacco leaves as previously described (Walter et al. 2004). Tobacco leaves were assayed for fluorescence 72 h after infiltration for subcellular localization.

Protoplasts were isolated from 10-day-old wheat seedlings. Young leaves and sheaths were chopped and submerged in an enzyme solution [1% cellulose R-10 (Yakult Honsa Co. Ltd., Tokyo, Japan), 0.25% macerozyme R-10 (Yakult Honsa Co. Ltd.), 0.1% BSA, 10 mM MES, 500 mM mannitol, and 1 mM CaCl2] for 6 h with gentle shaking. The samples were then filtered through a 40-μm nylon mesh into 50-mL conical tubes and centrifuged for 4 min at 300 × *g* to remove the enzyme solution. The pelleted protoplasts were resuspended with equal volumes of a W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM MES) and incubated for 10 min with gentle shaking. The protoplasts were further incubated on ice for at least 4 h and resuspended in an MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4.7 mM MES). Then, each of the constructed plasmids was transfected into the protoplasts using a 40% polyethylene glycol (PEG) solution [40% PEG, 400 mM mannitol, and 100 mM $Ca(NO_3)_2$] for 30 min at room temperature. After 16 h of incubation, the transfected protoplasts were observed under a confocal microscope. Tobacco leaves and wheat protoplasts cells were subjected to confocal laser-scanning microscopy (LSM 700; Carl Zeiss, Oberkochen, Germany).

Phylogeny analysis

The HUB2 protein sequences from seven different species were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein/). A phylogenetic tree was generated using MEGA-X software (Kumar et al. 2018).

Genetic and physical mapping

The physical locations of the TaHUB2 genes in wheat and on the chromosomes were obtained by BLASTn searches against the IWGSC Refseq v1.0 (https://wheat-urgi.versailles.inra.fr/) genome database. Graphical map drawing was performed using MapChart software (Voorrips 2002).

In vitro ubiquitination assay

The in vitro ubiquitination assay was performed as described previously (Lim et al. 2013; Park et al. 2018). The full-length *TaHUB2* sequence was cloned into a pMAL-c5x vector (NEB). The MBP was expressed by BL21 (DE3) pLysS (Promega Corporation, Madison, WI, USA) and purified using amylose resin (NEB). The reaction contained 6X His-tagged *Arabidopsis* UBC1 and UBC2, ubiquitin (Sigma-Aldrich, St. Louis, MO, USA), human E1 (Sigma-Aldrich), and ubiquitination z-buffer (1 M Tris-HCl, pH 7.5; 40 mM ATP; 100 mM $MgCl_2$; 40 mM DTT). This reaction mixture was incubated at 30°C for 3 h and then subjected to 11% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE); subsequently, the reaction products were transferred to a nitrocellulose membrane. Ubiquitination analysis was conducted using an anti-ubiquitin antibody (Sigma-Aldrich) and a secondary goat anti-rabbit IgG peroxidase antibody (Sigma-Aldrich).

Yeast two-hybrid assay

The Matchmaker GAL4-bases two-hybrid system (Clontech Laboratories, Inc., Mountain View, CA, USA) was used to perform the yeast two-hybrid assays. Entry clones containing full-length TaHUB2 were recombined into the pGDAT7 vector (Clontech Laboratories, Inc.) to generate activation domain (AD) constructs using a T4 DNA ligation system (NEB). The entry clones containing full-length TaH2B and *Arabidopsis* UBC1 and UBC2 were recombined into the pGBKT7 vector (Clontech Laboratories, Inc.) to generate binding domain (BD) constructs. Each construct and empty vector was transformed into the AH109 yeast strain and then co-transformed and grown in D-aspartate oxidase (DDO) medium. After 5 days, each clone was cultured in DDO broth and spotted on a quadruple dropout (QDO) medium to test for protein interactions.

In vitro pull-down assay

To observe the protein–protein interactions between the TaHUB2 RING finger protein and the TaH2B proteins, in vitro pull-down assays were performed as described by (Park et al. 2018). Each His-tagged TaH2B protein and MBP-tagged TaHUB2 RING finger protein was expressed in the *E. coli* strain BL21. After sonicating each of the MBP-tagged proteins, they were incubated with or without the His-tagged H2B proteins using a Pierce™ His Protein Interaction Pull-Down Kit (Thermo Fisher Scientific). The reaction was terminated by the addition of 6× SDS sample buffer followed by 5 min of boiling at 95°C. The proteins were then separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immune signal detection was carried out using an anti-His antibody (Sigma-Aldrich) and anti-MBP antibody (NEB).

Cell-Free degradation assays

Cell-free degradation assays were conducted as described by (Byun et al. 2017). Cell-free protein crude extracts were prepared from non-vernalization or 50 days vernalization wheat seedlings using a protein extraction buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, and protease inhibitor cocktail [Roche, Basel, Switzerland]). Bacterially expressed MBP-TaHUB2 after 6 h at 30°C. Each sample was harvested, boiled, separated by 10% SDS–PAGE, and analyzed by immunoblotting using an anti-MBP antibody. Rubisco was used as a loading control.

Histone H3 methylation analysis

Nuclear protein was isolated using a subcellular protein fractionation kit for tissues (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 100 mg of ground sample was mixed with an ice-cold cytoplasmic extraction buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific), incubated (4°C, 10 min), and centrifuged

(500 × *g*, 5 min); the supernatant provided the cytosolic fraction, whereas the pellet, which contained the membrane and nuclear fractions, was resuspended in an ice-cold membrane extraction buffer containing protease inhibitors (Thermo Scientific), incubated (4°C, 10 min), and centrifuged (3000 × *g*, 5 min). The resultant supernatant provided the membrane fraction, which was resuspended in an ice-cold nuclear extraction buffer containing protease inhibitors (Thermo Scientific), incubated (4°C, 30 min), and centrifuged (5000 × *g*, 5 min). The resultant supernatant provided the nuclear protein.

For immunoblot analysis, 10 mg protein was separated on a 13% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by electroblotting. The membranes were hybridized with specific antibodies, such as anti-histone H3 (ab18521; Abcam, Cambridge, MA, USA), anti-H3K4me3 (Cell Signaling Technology, Danvers, MA, USA), anti-H3K36me3 (Cell Signaling Technology), and anti-ubiquityl-histone H2B (Cell Signaling Technology). The signal was detected and visualized using a chemiluminescence system.

Results

Isolation and sequence analysis of TaHUB2 in wheat

In the present study, to characterize the putative RING-type E3 ligase *TaHUB2*, we conducted BLAST searches against the wheat genomic sequence URGI (http://wheat-urgi.versailles.inra.fr) using the amino acid sequences of the AtHUB2 (AT1G55250.1) protein and obtained the gene predicted to be TaHUB2 from 11 protein sequences (Supplementary Figure S1). Among these, three protein sequences (TraesCS3D02G466100, TraesCS3A02G467300, and TraesCS3B02G511800) with high levels of sequence similarity were selected using Ensembl Plants (http://plants.ensembl.org/Multi/Tools/Blast). These proteins were highly conserved in the amino acid sequences, and TraesCS3A02G467300 (TaHUB2) was selected (Supplementary Figure S2). The full-length cDNA of TaHUB2 was 2532 bp in size and encodes 844 amino acids. Sequence alignment analysis indicated that wheat TaHUB2 contains a conserved RING-C3HC4 domain (residues 792–830) and one nuclear localization sequence (NLS) (residue 746–756) via the cNLS Mapper (http://nls-mapper.iab.keio.ac.jp) (Figure 1(A)). Multiple sequence alignment showed that TaHUB2 has 57% sequence identity with AtHUB2. However, the rice HUB2 ortholog gene (Os10t0565600, named FRRP1) shared 76% sequence identity with TaHUB2, whereas *Brachypodium distachyon* (BRADI_2g33230v3) and *Hordeum vulgare* (HORVU3Hr1G099950) were 84% and 94% identical to TaHUB2 (Figure 1(B)). All of these predicted HUB2 proteins contained a single RING-C3HC4 domain in the C-terminal region. Therefore, the cloned TaHUB2 is a putative *Arabidopsis* AtHUB2 orthologue in wheat.

TaHUB2 e3 ligase interacts directly with the TaH2B protein

TaHUB2 contains an NLS domain (Figure 1(A)). To confirm the localization of TaHUB2, which included the NLS domain, we fused TaHUB2 with C-terminus GFP (pK7FWG) and transiently expressed them in a wheat protoplast using a PEG transfection system. Interestingly, the GFP signals in the wheat protoplast cell showed localization not only in the nucleus but also in the cytoplasm (Figure 2(A)).

More recently, it has been shown that GhHUB2 RING finger E3 ligase interacts with histone GhH2B1 in cotton (Feng et al. 2018; Chen et al. 2019). We therefore cloned *TaH2B*, which is a wheat ortholog of *Arabidopsis* AtHTB11 (AtH2B) (Bergmüller et al. 2007). To examine whether wheat TaH2B directly interacts with TaHUB2, we performed co-transformation in yeast. The TaHUB2-AD and TaH2B-BD cells could grow on DDO and QDO media (Figure 2(B)). Next, we conducted an in vitro pull-down assay using TaHUB2 and the His-tagged TaH2B protein. The bound protein was co-incubated with HisPur Cobalt resin. The in vitro pull-down assay showed that the TaHUB2 protein was detected and pull downed by TaH2B (Figure 2(C)). Furthermore, using BiFC assays with tobacco leaves, we also identified TaH2B and TaHUB2 interactions in plants. The pGTQL1211 (N-YFP) and pGTQL1221 (C-YFP) vectors were co-transformed in *Nicotiana benthamiana* leaf cells. Interestingly, nYFP-TaH2B and cYFP-TaHUB2 were expressed in the nucleus (Figure 2(D)). Next, we confirmed the transient expression of TaH2B in tobacco leaves and found that the TaH2B protein was localized in the nucleus (Figure 2(E)). These findings indicated that TaHUB2 E3 ligase directly interacts with TaH2B in wheat.

TaHUB2 interacts with UBC1 and UBC2 and has ubiquitin ligase activity

To determine whether the TaHUB2 protein has E3 ligase activity and is capable of interacting with UBCs in vitro, we first checked the interaction of TaHUB2 with UBC1 and UBC2 using a yeast two-hybrid assay. This assay found that TaHUB2 directly interacted with UBC1 and UBC2 (Figure 3(A)). Furthermore, we expressed a recombinant TaHUB2 protein fused in an MBP-tagged vector (pMAL-c5x), which also generated an MBP-TaHUB2^{C812A} (the fourth cysteine was replaced by arginine in the RING domain) and *Arabidopsis* UBCs (UBC1, UBC2, UBC8, UBC9, UBC10, UBC11, and UBC19). The purified pMAL-c5x-TaHUB2 protein was incubated in the presence or absence of E1 and E2 and was then subjected to an anti-ubiquitin antibody. The results showed that a ubiquitination chain was observed only in the presence of E1, UBC1 or UBC2 (E2), and TaHUB2 (E3) (Figure 3(B) and Supplementary Figure S3). However, in the absence of E1, E2, or E3 and in the presence of MBP-TaHUB2^{C812A}, the TaHUB2 protein showed no ubiquitination activity (Figure 3(B)). Next, to examine whether the TaHUB2 E3 ligase ubiquitinates the histone H2B protein, we performed a ubiquitination assay by incubating MBP-TaHUB2 with H2B and western blotting using an anti-His antibody. The in vitro ubiquitination assay revealed a monoubiquitinated H2B band when E1, E2, and E3 were present, whereas monoubiquitinated H2B was not detected in the absence of E1, E2, or E3 (Figure 3(C)).

The relative expression of TaHUB2 and its methylation level are reduced upon increasing the vernalization treatment time

In *Arabidopsis*, the *FLC*-mediated repression of the initiation of flowering is influenced by vernalization, which stimulates

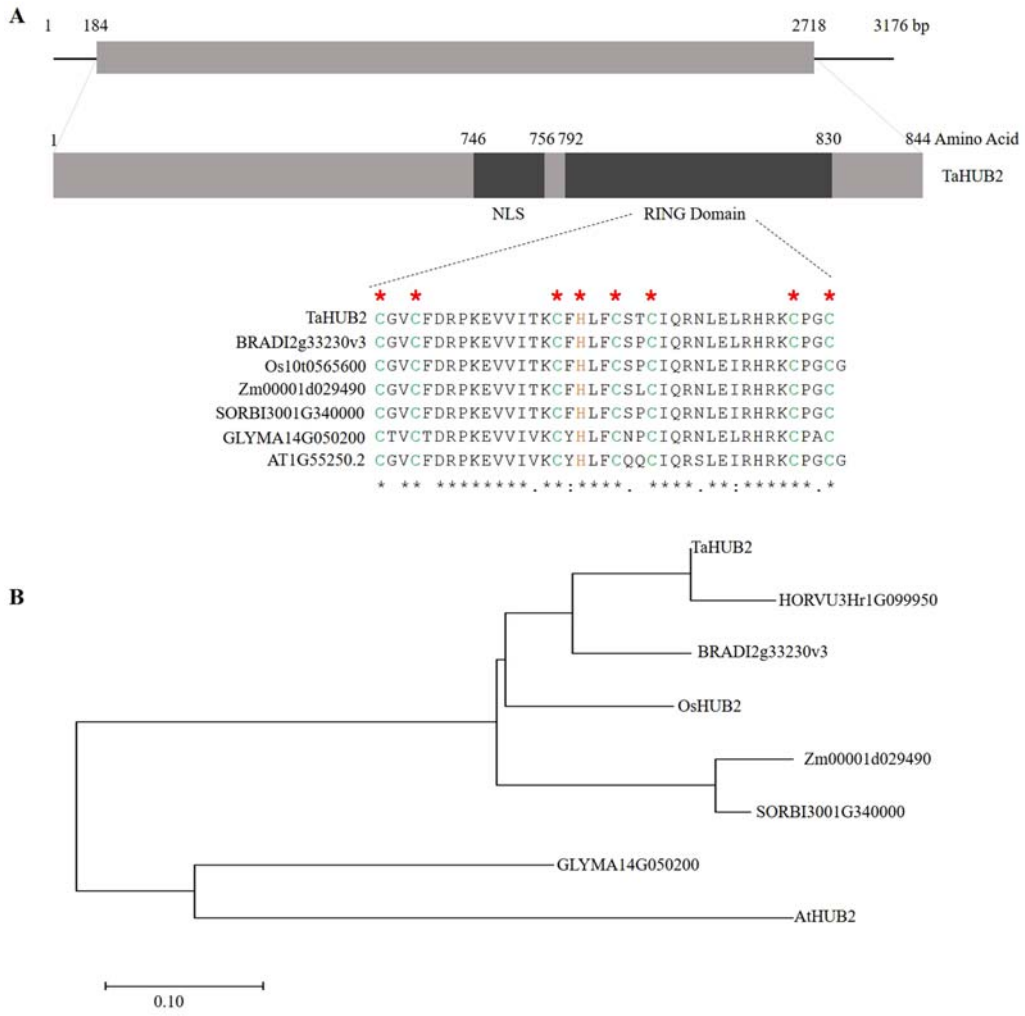

Figure 1. Identification and characterization of TaHUB2 in wheat. (A) Schematic diagram of full-length TaHUB2 cDNA. The solid bar depicts the coding region. The solid lines represent the 5′- and 3′-untranslated regions. (Lower) Schematic structure of TaHUB2. The nuclear localization sequence (NLS) and RING motif are shown as dark gray bars. The multiple alignment of TaHUB2 and other orthologs of RING-C3HC4-type HUB2 proteins, such as those from *Zea mays* (ZM), *Triticum aestivum* (TRAES), *Hordeum vulgare* (HORVU), *Oryza sativa* (OS), *Arabidopsis thaliana* (AT), *Brachypodium distachyon* (BRADI), and *Sorghum bicolor* (SORBI). The alignment was performed using Clustal2 software (http://ebi.ac.uk/clustalw/). (B) Phylogenetic tree showing the deduced protein sequence of TaHUB2 and other amino sequences from several plants.

the transition to a reproductive stage (Sheldon et al. 1999; Deng et al. 2015). To explore the possible roles of TaHUB2 in different wheat vernalization periods, we first examined the gene expression pattern of TaHUB2 by qRT-PCR using plants at four leaf stages. Plant samples were collected from 0, 10, 20, 30, and 50 days vernalized plants at four leaf stage seedling. *TaHUB2 e*xpression was the highest at 0 day of vernalization in cv. *Keumkang*. However, as the vernalization period increased (10, 20, 30, and 50 days), *TaHUB2* expression was gradually decreased from 10 to 50 days of vernalization in *Keumkang* (Figure 4(A)). Some abiotic stress conditions affect not only the transcript level but also the protein stability (Byun et al. 2017). Because the transcript level of *TaHUB2* was decreased when vernalization started, we wanted to confirm the possibility that the TaHUB2 protein level might also be affected by vernalization. To verify this hypothesis, we performed an in vitro cell-free degradation assay. The MBP-TaHUB2 recombinant protein was co-incubated for 0, 3, and 6 h with or without 50 days vernalized wheat protein using plants at four leaf stages. The protein level of MBP-TaHUB2 was significantly reduced over time (0–6 h) under 50 days vernalized conditions compared to that under normal conditions (Figure 4(B)). Various environmental conditions affect histone methylation and/or acetylation during H2B ubiquitylation (H2Bub1) and influence histone modification (Hwang et al. 2003; Yuan et al. 2013; Eom and Hyun 2018). To verify whether vernalization also has an effect on the histone H3 methylation level, we conducted western blot analysis in the absence or presence of the vernalization treatment of four leaf stage seedlings using H3K4me3 and H3K36me3 antibodies (Figure 4(C)). The levels of histone H3 methylation (H3K4me3 and H3K36me3) and H2Bub1 were significantly reduced under vernalization treatment.

The transcription levels of VRN genes are regulated by vernalization treatment

We evaluated the expression of flowering-related genes such as *VRN1*, *WFT*, and *VRN2* (*ZCCT1* and *ZCCT2*) in the *Keumkang* wheat variety vernalized for 0, 10, 20, 30, and 50 days at 4°C. The transcription level of *VRN2* (ZCCT1 and ZCCT2) was significantly reduced under vernalization for 50 days starting after 10 days of vernalization. Conversely, the transcription level of *VRN1* increased slightly in the *Keumkang* wheat variety as the duration of vernalization increased; in contrast, the transcript level of WFT was highly increased after 50 days of vernalization in the *Keumkang*

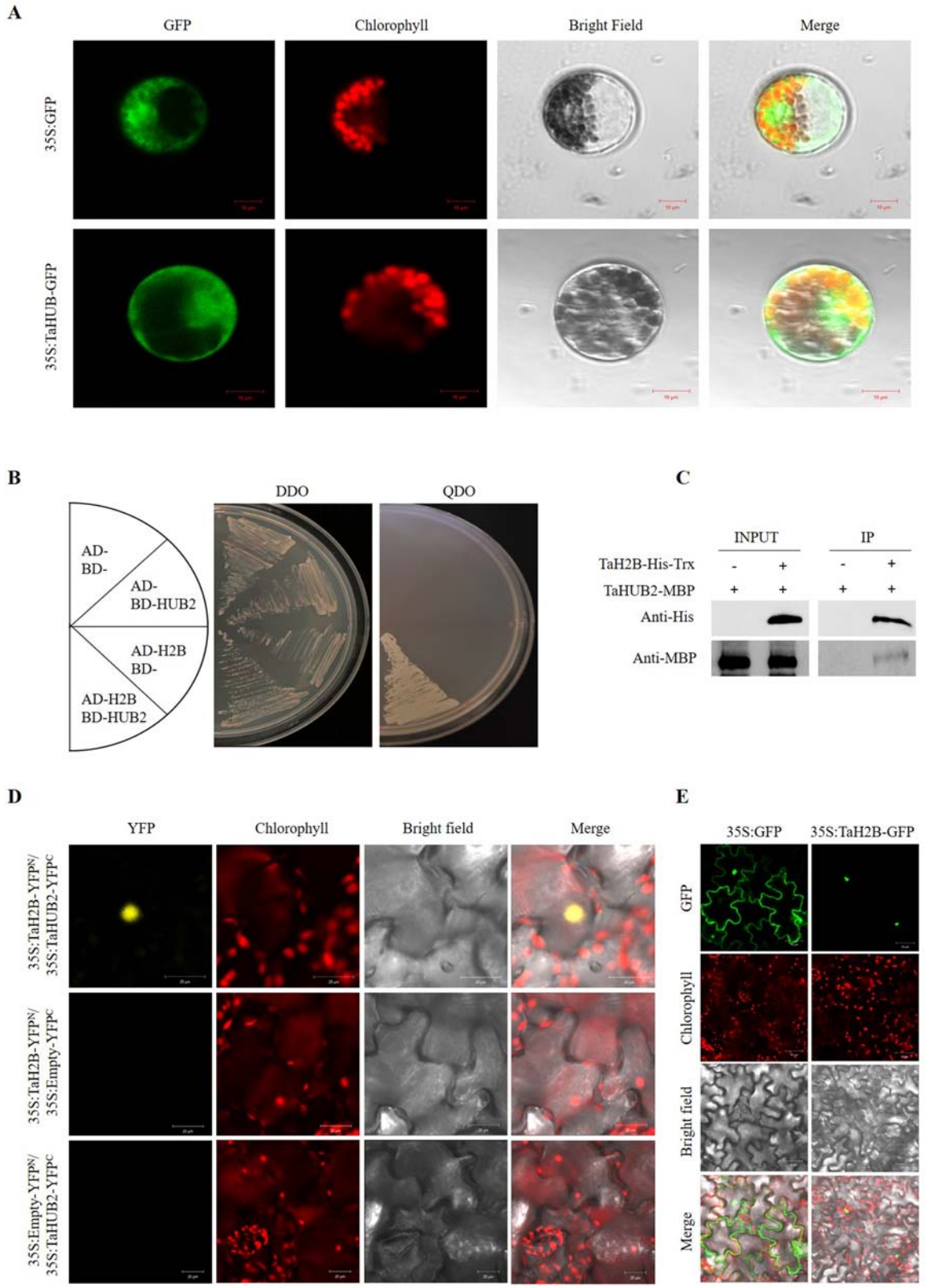

Figure 2. Wheat HUB2 E3 ligase physically interacts with H2B. (A) Subcellular localization of 35S::GFP-TaHUB2 in wheat protoplasts. (B) Yeast two-hybrid assay with HUB2 and H2B. The full-length TaHUB2 was cloned into pGADT7, and TaH2B was cloned into pGBKT7. Each of the constructions was co-transformed into AH109 yeast strain in SD/-Leu/-Trp and SD/-His/-Leu/-Trp medium. (C) In vitro pull-down assay of MBP-TaHUB2 and TaH2B-His-Trx proteins. (D) BiFC assay of HUB2 and H2B interactions. The vector constructs of the 35S promoter and the N-terminal YFP/C-terminal YFP. H2B fused into the N-terminal YFP, and HUB2 fused into the C-terminal YFP. (E) Subcellular localization of 35S::GFP-TaH2B in tobacco leaves via *Agrobacterium*-mediated infiltration.

wheat variety (Figure 5). These results demonstrated that vernalization affects the H3K4me3, H3K36me3, and H2Bub1 levels in wheat as shown in Figure 4, might be resulted in a reduction of *VRN2* transcript level and in turn, an increase in the level of *VRN1*, and WFT which subsequently leading to a heading stage after vernalization.

Discussion

Recent studies have suggested that *Arabidopsis* and rice histone H2B monoubiquitination enzyme HUBs are involved in controlling plant growth and development (Liu et al. 2007; Cao et al. 2008, 2015; Bourbousse et al. 2012; Du et al.

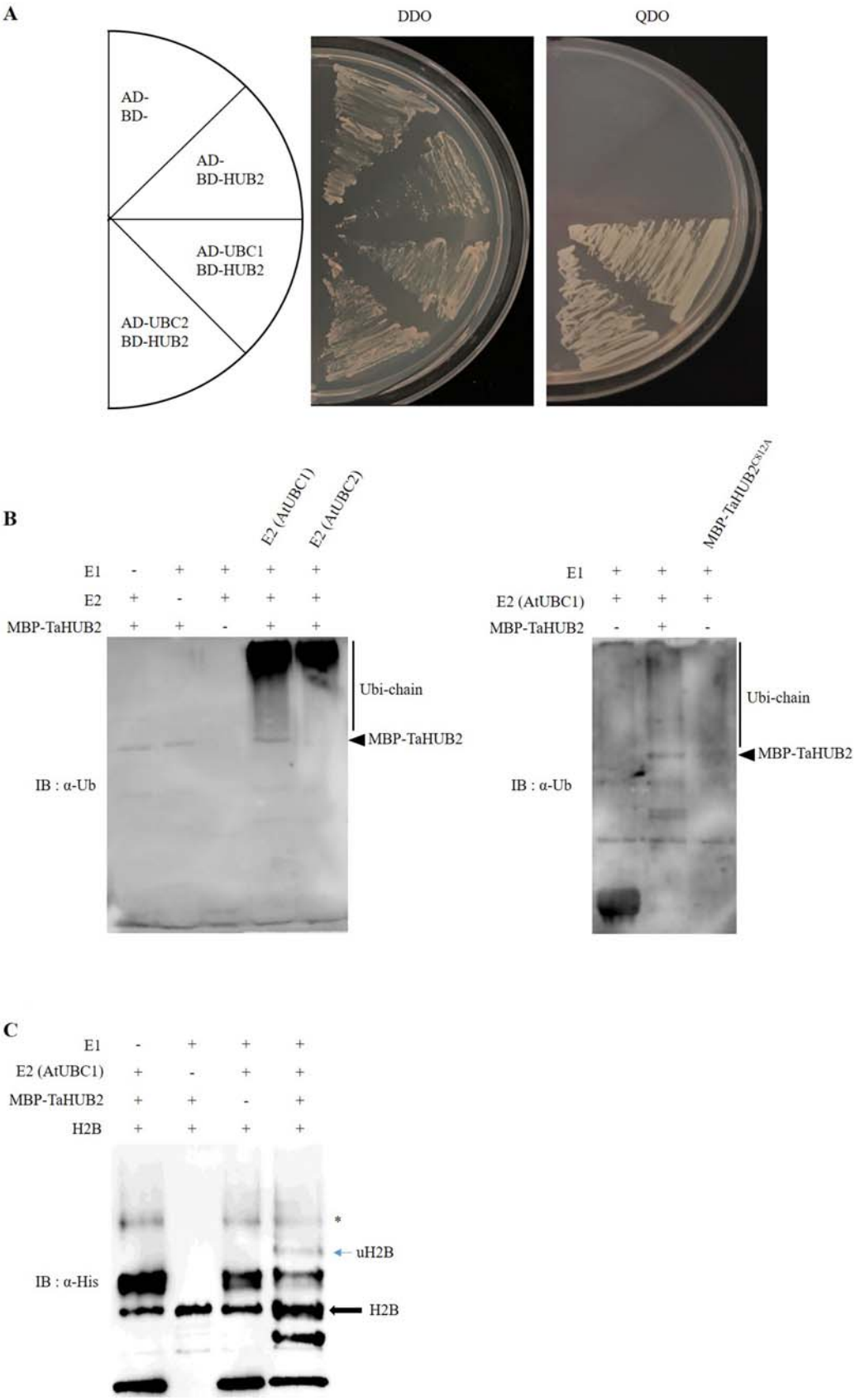

Figure 3. In vitro ubiquitination of TaHUB2 with H2B as a substrate. (A) Yeast two-hybrid assay with TaHUB2 and AtUBC1 and AtUBC2. The full-length TaHUB2 was cloned into pGADT7, and AtUBC1 and AtUBC2 were cloned into pGBKT7. Each of the constructions was co-transformed into the AH109 yeast strain in SD/-Leu/-Trp and SD/-His/-Leu/-Trp mediums. (B) Auto-ubiquitination activity of TaHUB2. The ubiquitination reaction contains E1 (human), E2 (*Arabidopsis* UBC1, UBC2), E3 (MBP-TaHUB2 and MBP-TaHUB2^{C812A}), ubiquitin (Ub), and ATP. Polyubiquitin chains were observed with immunoblotting using a Ubi-antibody. (C) In vitro ubiquitination assay of His-tagged H2B proteins with MBP-TaHUB2 E3 ligase. H2B protein was incubated with the MBP-TaHUB2 protein, E1 (human), and UBC1 (*Arabidopsis*) for 3 h. Immune signals were detected using an anti-His antibody.

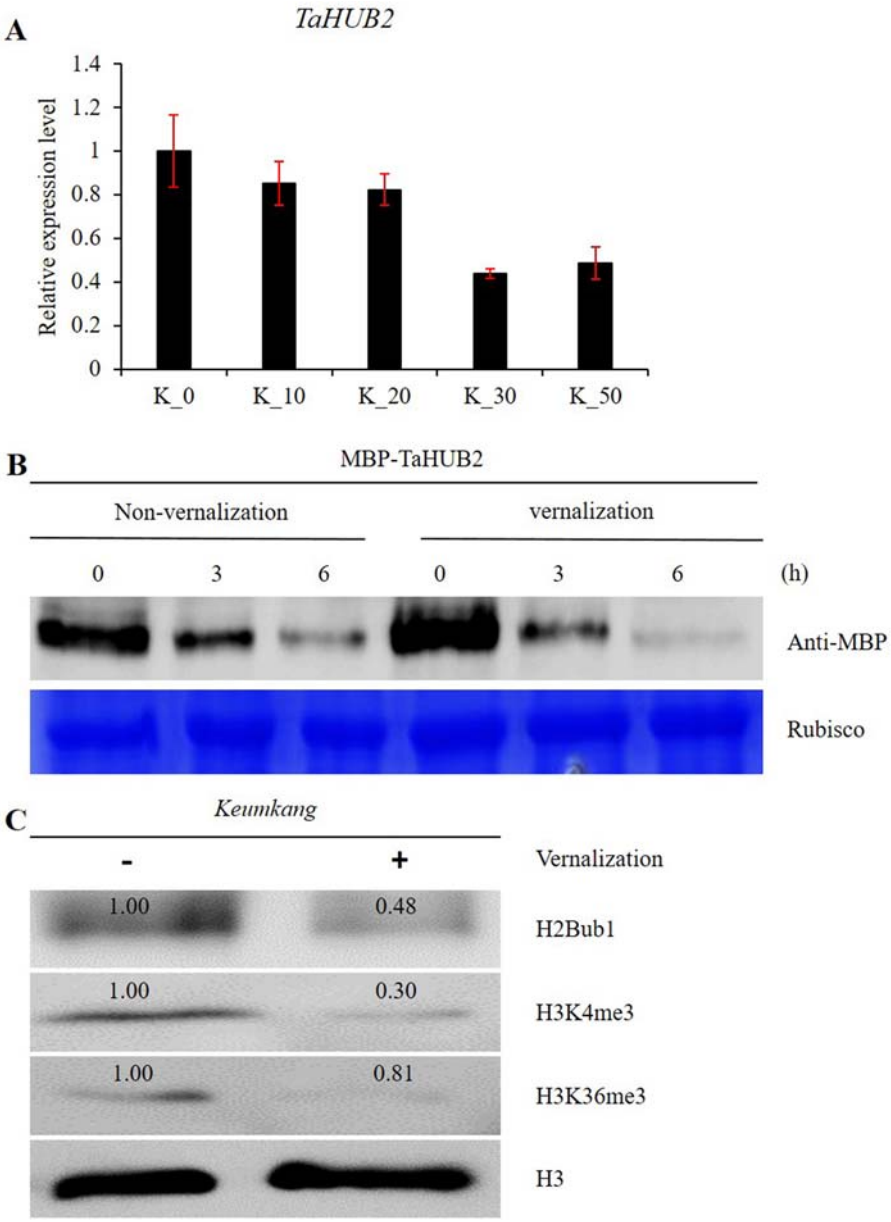

Figure 4. The relative expression of *TaHUB2* and its methylation level were reduced upon increasing the vernalization treatment period. (A) TaHUB2 transcripts quantified by qRT-PCR in the different wheat vernalization period samples. Each bar represents the mean ± SD of three independent experiments. In vitro cell-free degradation assays of TaHUB2 under non-vernalization and vernalization conditions. (B) In vitro cell-free degradation assays of TaHUB2 under non-vernalization and vernalization conditions. (C) The H2Bub1, H3K4me3, and H3K36me3 levels detected by western blot under vernalization treatment. (–): vernalization for 0 days, (+): vernalization for 50 days. The H3 level was detected as a loading control.

2016; Chen et al. 2019). Nevertheless, the wheat HUB has not been characterized to a great extent so far. In the present study, functional analysis of *TaHUB2 gene* in wheat were conducted. We isolated the candidate wheat (*TaHUB2*) orthologue of *Arabidopsis AtHUB2* (Supplementary Figures S1 and S2). We further confirmed the phylogenetic analysis of the TaHUB2 amino acids that revealed its close resemblance to rice OsHUB2 (Figure 1(B)), which has been previously reported to play a critical role in flowering time and anther development (Cao et al. 2015; Du et al. 2016).

The RING-type E3 ligases, which mediate the ubiquitination pathway, play various roles in plant development including flowering time (Cao et al. 2008). The conserved RING domain forms a single subunit that combines with E2 ubiquitin and the substrate, which is necessary for E3 ligase activity (Stone et al. 2005). Most recent reports have shown that HUB2 has a ring domain and mediates E3 ligase activity *via* the ubiquitination pathways in plants (Du et al. 2016; Feng et al. 2018). We therefore checked the TaHUB2 protein sequence and confirmed that TaHUB2 also contains a RING domain similar to that in HUB2 proteins in other plant species (Figure 1(A)). Most of HUB2 proteins had RING-C3HC4 (RING-HC) types. In plants, HUB and most RING-HC type proteins are localized in the nucleus and cytoplasm (Cao et al. 2008, 2015; Sun et al. 2019). Wheat TaHUB2 also contains an NLS domain (Figure 1(A)), and we therefore confirmed the localization of TaHUB2 in the nucleus and cytoplasm (Figure 2(A)). We hypothesized that TaHUB2 recognizes specific substrate proteins via the RING domains through the ubiquitination pathway and that TaHUB2 may interact with substrates not only in the nucleus but also in the cytoplasm.

Molecular mechanism in flowering time has been strictly controlled by both endogenous and environmental signals, such as vernalization, photoperiodic, autonomous and gibberellin pathways (Cho et al. 2017). Numerous genes are

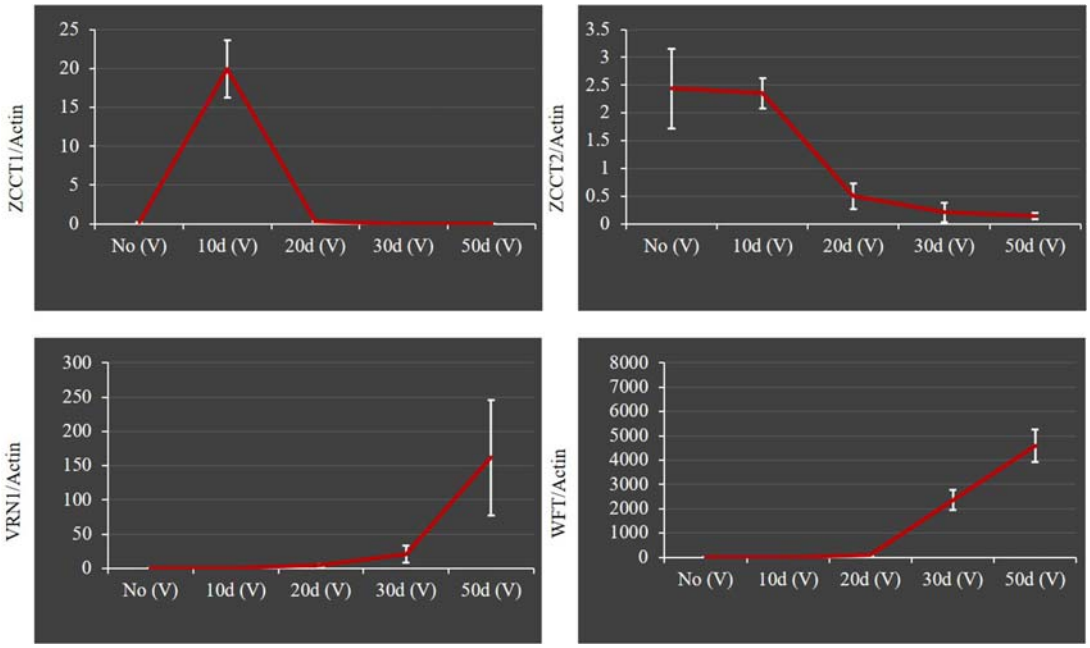

Figure 5. The transcription level of the VRN genes is regulated by vernalization treatment. *TaVRN1, TaWFT, TaZCCT1,* and *TaZCCT2* transcripts quantified by qRT-PCR in the different wheat vernalization period samples. Each bar represents the mean ± SD of three independent experiments.

associated these genetic pathways and closely linked together. Among them, vernalization requirements for a period of low temperature exposure that will boost the transition from the vegetative to the reproductive stage (González et al. 2002; Slafer et al. 2015; Porker et al. 2016). However, in crops, little is known about the genes regulated by vernalization.

We therefore analysis to investigate the function of *TaHUB2* during vernalization fulfillment using different vernalization period samples of *Keumkang* wheat varieties (Figure 4(A)). Moreover, Ubiquitin E3 ligases can self-catalyzed themselves, thereby targeting their own in negative feedback loop (de Bie and Ciechanover 2011; Byun et al. 2017). TaHUB2 exhibited self-ubiquitination activities (Figure 3(B)) and was degraded in cell-free extracts (Figure 4(B)). In our previous study, we found that the heading time was not differ between spring and winter types if they were vernalized about 50 days (Kim et al. 2021). Therefore, 50 days of vernalization is enough to synchronize heading time regardless of vernalized requirement. This degradation of TaHUB2 was more rapid when 50 days vernalized than non-vernalized. Notably, the production of transcription and protein level of *TaHUB2* was negatively responsive to vernalization in accordance with the role of *HUB genes* in other plant species (Cao et al. 2008; Du et al. 2016).

Chromatin regulation play fundamental roles in plants that are involved in gene regulation via various responses (Deal and Henikoff 2011). *Arabidopsis* monoubiquitinated H2B (H2Bub1) was recently reported to accelerate *FLC* transcription via the methylation of H3 lysine residues (Cao et al. 2008). We verified physical interactions between TaHUB2 and TaH2B from wheat (Figure 2). Previous studies reported that a number of proteins interact with RING type E3 ligase as substrates (Lim et al. 2013; Hwang et al. 2017; Park et al. 2018). Similarly, TaHUB2 ubiquitinates wheat Histone H2B leading to monomer chain form *via* 26s proteasome pathway (Figure 3). Therefore, these results support the TaH2B is a target substrate protein of TaHUB2 RING-type E3 ligase. The function of histone variants has been better understood by the improved insight studies that show how expression levels vary depending on the cell cycle or stage of development (Buschbeck and Hake 2017; Jiang et al. 2020). Compared with histone H3 and H2A, an expended set of H2B rules have been reported in *Arabidopsis* (Talbert et al. 2012). The histone *H2B* genes were specifically expressed in reproductive tissues and classified highly divergent in flowering plants, which has been deposited euchromatic and heterochromatic regions in *Arabidopsis* (Jiang et al. 2020). We therefore tested the effect of ubiquitylated H2B (H2Bub1) in vernalized plants, and our results demonstrated that the levels of H2Bub1 were significantly reduced after vernalization (Figure 4(C)). This finding suggested that innate function of TaH2B has been related to the post translational modification in accordance with ubiquitination and flowering pathways in wheat.

As recently reported, the expression of *FLC* is stimulated by the deposition of H3K4me3 by the *Arabidopsis* Paf1 complex, trxG proteins, and the integration of H2A.Z by the SWR1-like complex (He et al. 2004; Deal et al. 2007; March-Díaz et al. 2007, 2008; Lázaro et al. 2008; Tamada et al. 2009), and the loss of the Paf1 complex, trxG proteins, the SWR1-like complex, or H2A.Z results in reduced *FLC* expression and promotes early flowering (Deal and Henikoff 2011). We therefore tested the transcription level of VRN genes in wheat. The transcript level of VRN2 genes (ZCCT1 and ZCCT2) were significantly downregulated after vernalization, whereas VRN1 (AP1) was slightly increased in the *Keumkang* wheat variety with an extended period of 50 days of vernalization. WFT (VRN3) was highly expressed after 50 days of vernalization in the *Keumkang* wheat variety (Figure 5). These results indicated that the decrease in histone H3 methylation after vernalization results in the downregulation of VRN2 gene expression, which subsequently leads to heading or flowering in wheat. Further investigations are required to elucidate the genetic interactions between *VRN2* and *TaHUB2* in wheat.

Conclusion

In this study, our results provided evidence that TaHUB2 acts as an ubiquitin RING-type E3 ligase in wheat. The interaction between TaHUB2 and TaH2B might be important to understanding histone modification in wheat. Gene expression and protein level also suggested that *TaHUB2* are involved in vernalization pathways, and may also be required for heading in wheat. However, further investigations are still needed to demonstrate the mechanism and function of TaHUB2 in the process of vernalization.

Author contributions

JHK, IUK, MSK, and YWS conceived and designed the experiments. JHK, IUK, and MSK performed the experiments. JHK, IUK, MSK, and YWS analyzed the data and wrote the paper. All authors reviewed and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by a Korea University grant and Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (2017R1D1A1B06030349).

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