Heat Shock Transcription Factor B2b Acts as a Transcriptional Repressor of Vin3, a Gene Induced by Long-term Cold for Flowering

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HEAT SHOCK TRANSCRIPTION FACTOR B2b Acts as a Transcriptional Repressor of *VIN3*, a Gene Induced by Long-Term Cold for Flowering

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Abstract

Vernalization, an acceleration of flowering after long-term winter cold, is an intensively studied flowering mechanism in winter annual plants. In *Arabidopsis*, Polycomb Repressive Complex2 (PRC2)-mediated suppression of the strong floral repressor, *FLOWERING LOCUS C* (*FLC*), is critical for vernalization and a PHD finger domain protein, VERNALIZATION INSENSITIVE 3 (VIN3), recruits PRC2 on *FLC* chromatin. The level of *VIN3* was found to gradually increase in proportion to the length of cold period during vernalization. However, how plants finely regulate *VIN3* expression according to the cold environment has not been completely elucidated. As a result, we performed EMS mutagenesis using a transgenic line with a minimal promoter of *VIN3* fused to the *GUS* reporter gene, and isolated a mutant, *hyperactivation of VIN3 1* (*hov1*), which showed increased GUS signal and endogenous *VIN3* transcript levels. Using positional cloning combined with whole-genome resequencing, we found that *hov1* carries a nonsense mutation, leading to a premature stop codon on the *HEAT SHOCK TRANSCRIPTION FACTOR B2b* (*HsfB2b*), which encodes a repressive heat shock transcription factor. HsfB2b directly binds to the *VIN3* promoter, and *HsfB2b* overexpression leads to reduced acceleration of flowering after vernalization. Collectively our findings reveal a novel fine-tuning mechanism to repress undesirable vernalization responses.

Introduction

As sessile organisms, plants evolve to adapt to their surrounding environment. As the transition from the vegetative to reproductive phase is usually irreversible, the proper decision of flowering time in response to the environment is one of the most important developmental processes in plants[1]. Vernalization, an acceleration of flowering after long-term winter cold, is one of the mechanisms that render plants to flower in a timely manner. *Arabidopsis* winter annuals exhibit a late-flowering phenotype but their flowering time is dramatically accelerated by vernalization. In contrast, summer annuals exhibit an early-flowering phenotype regardless of cold treatment[2]. Before winter, the winter annuals display strong expression of *FLOWERING LOCUS C* (*FLC*), a MADS-box transcription factor that represses precocious flowering, however, *FLC* is gradually suppressed according to the winter cold period, which allows plants to flower in the spring[2-4]. Thus, the molecular mechanism of vernalization in *Arabidopsis* involves the suppression of *FLC* by winter cold. Suppression of *FLC* during vernalization involves epigenetic silencing, and requires a protein complex called the PHD-PRC2 complex, which includes the core components of PRC2, CURLY LEAF (CLF), an *Arabidopsis* homologue of H3K27 methyltransferase, SWINGER (SWN), VERNALIZATION 2 (VRN2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE), the WD-40 domain protein, MSI1, and VERNALIZATION INSENSITIVE3 (VIN3), a protein bearing a Plant Homeo Domain (PHD) motif[5,6]. PHD-PRC2 mediates *FLC* silencing by accumulating H3K27me3, a repressive histone modification, on *FLC* chromatin[7,8]. Among the genes encoding the PHD-PRC2 complex, *VIN3* is the only gene induced by vernalization and its expression gradually increases proportionally to the length of the cold period, which is an uncommon aspect[8]. However, the regulatory mechanism of *VIN3* in vernalization remains elusive.

Cellular proteins are easily damaged when exposed to various environmental stresses. To protect cellular proteins from such cellular stresses, most eukaryotic organisms, including plants, have evolved molecular chaperones[9]. The most well-studied molecular chaperones are heat shock proteins (HSPs), which are induced by myriads of cellular stresses as well as heat shock[10]. The activation of HSPs, a general stress response in most eukaryotic organisms, is induced by a family of transcription factors known as Heat Shock Factors (Hsfs). Hsfs act as components of signal transduction that induce the expression of *HSPs* in response to a broad range of abiotic stresses[11]. By binding to the cis-element, called Heat Shock Elements (HSE; inverted repeat of a basal element 5'-nGAAn-3'), which are conserved in the promoters of heat stress–inducible genes of all eukaryotes, Hsfs directly regulate the transcription of stress-responsive genes, including HSPs[12-14]. There are 21 *Hsf* genes in the *Arabidopsis* genome and are divided into three classes; A, B and C[15,16]. Class A contains the motif (AHA motif) with activation activity, which is characterized by aromatic, large hydrophobic, and acidic amino acid residues[16]. Class A proteins have been reported to act as positive regulators in response to a broad range of stress conditions in plants[13,17,18]. In contrast, class B and C proteins are considered transcriptional repressors, as they lack AHA motifs and contain the repressive R/KLFGV motif[19]. Among the five class B proteins, HsfB1 and HsfB2b have been reported to act as transcriptional repressors, but positively regulate redundantly the acquired thermotolerance, an enhanced thermotolerance by prior heat treatment[20]. Besides the acquired thermotolerance, HsfB1 and HsfB2b have been shown to negatively regulate pathogen resistance redundantly[21], while HsfB2b alone has been shown to mediate abiotic stress responses of the circadian clock[22].

In the present study, we identified *HsfB2b* as a novel repressor of *VIN3*. Further, we isolated one mutant, *hov1,*

with hyperactive *VIN3* from a mutant pool that originated from the *pVIN3::GUS* reporter lines. The mutant was identified to carry a nonsense mutation in exon 1 of *HsfB2b*. Overexpression of *HsfB2b* rescued hyperactive *VIN3* in *hov1,* and HsfB2b was found to bind to the conserved HSEs located in the 5'-UTR of *VIN3*. Moreover, overexpression of *HsfB2b* in the late-flowering *FRI* Col background resulted in defects in the vernalization response, suggesting that *HsfB2b* negatively regulates the vernalization response.

Results

Isolation and characterization of the vernalization hypersensitive mutant, *hov1*

To identify upstream regulators of *VIN3*, the GUS reporter line (-0.2kb *pVIN3_U_I::GUS*)[23] was mutagenised with ethyl methanesulfonate (EMS). A total of 3,412 M1 lines were generated and plants showing enhanced GUS signals were isolated as hypersensitive mutants. One mutant was identified and initially named *hov1*. Compared to the parental line, which showed a very weak GUS signal after 3 days of cold exposure (3V), *hov1* showed an enhanced GUS signal with the same treatment (Fig. 1a). Consistent with the results of the GUS assay, endogenous *VIN3* transcript levels were enhanced in the mutant after 3V (Fig. 1b). The mutant phenotype was segregated by approximately 3:1 (81 WT vs. 29 mutants) when backcrossed with the parental line, which indicates that the mutant phenotype is caused by a mutation in a single locus. Thereafter, we performed a time course analysis of *VIN3* levels in *hov1* for vernalization treatment. As shown in Fig. 1c, *hov1* plants displayed higher levels of *VIN3* than the controls without vernalization, indicating that *HOV1* is necessary to completely suppress *VIN3* at room temperature. The *hov1* plants also had higher levels of *VIN3* throughout the vernalization time course and the mutant had higher levels of *VIN3* than the control plants after returning to room temperature for 5 days (40VT5). These results indicate that *HOV1* is required for the suppression of *VIN3* under all conditions.

To identify the causative mutation, *hov1* was crossed with L*er* for positional cloning. A total of 156 F2 plants with enhanced GUS signals were selected for mapping analysis. We mapped the mutation to the 590 kilobase pair interval on chromosome 4, which contained 142 genes (Supplementary Fig. S1). The genomes of *hov1* and parental *-0.2kb pVIN3_U_I::GUS* were sequenced using the Illumina sequencing method for comparison. Analysis of the sequence data revealed nine potentially disruptive point mutations, including one mutation within the At4g11660 gene (G to A, causing a nonsense mutation from Trp89 to the stop codon) (Fig. 1d). At4g11660 encodes the class B heat shock transcription factor, HEAT SHOCK TRANSCRIPTION FACTOR B2b (HsfB2b).

HsfB2b acts as a transcriptional repressor of *VIN3*

To verify that *HsfB2b* is the causative gene of the upregulation of *VIN3* level in *hov1*, we checked the *VIN3* level in the T-DNA-inserted mutant, *hsfb2b-1*. As expected, *hsfb2b-1* had higher levels of *VIN3* than wild-type Col-0 under non-vernalized conditions (Fig. 2a). Previously, *HsfB2b* was reported to display functional redundancy with *HsfB1* instead of *HsfB2a* for acquired thermotolerance, although the sequence of *HsfB2b* had higher homology with that of *HsfB2a* than *HsfB1*[20]. To determine whether *HsfB2b* is functionally redundant with *HsfB1* for the vernalization response, we compared *VIN3* levels among the *hsfb2b, hsfb1,* and *hsfb1 hsfb2b* mutants. The *hsfb1* mutant displayed similar levels of *VIN3* to the wild-type under all conditions. However, the *hsfb1 hsfb2b* double mutant did not show any difference compared with *hsfb2b* in *VIN3* levels (Fig. 2a). Such finding suggests that *HsfB1* is not functionally redundant to *HsfB2b*, at least for *VIN3* regulation.

Finally, we introduced *HsfB2b::HsfB2b-myc* into the *hov1* mutant to determine whether *HsfB2b* can rescue the *hov1* mutation. The transcript levels of *HsfB2b* were found to be overexpressed in all transgenic lines we obtained (Fig. 2c, Fig. 6a, and Fig. 6d). Here, we used two representative lines of *HsfB2b:HsfB2b-myc hov1,* #1,

and #2. As expected, the phenotype of the GUS signal in *hov1* was complemented by *HSFB2b::HSFB2b-myc,* such that the GUS signal was barely detected after 3 d of cold exposure (Fig. 2b). Moreover, the endogenous *VIN3* transcript levels in both transgenic lines were lower than those in Col, as well as the *hov1* mutant, whereas *HsfB2b* transcript levels in the transgenic lines were higher than those in Col (Fig. 2c and d). Taken together, our results indicate that *HsfB2b* is a causative gene that reduces *VIN3* level in *hov1* and acts as a transcriptional repressor of *VIN3*.

In a previous report, *VIN3* was found to gradually increase by vernalization from the first day of cold treatment[24]. Thus, we determined whether HsfB2b affects *VIN3* expression during the initial stage of vernalization (Fig. 2e and f). As shown, *hsfb2b* caused strong derepression of *VIN3* from the initial phase, and the effect was strongest after 3 h of cold treatment. Of note, the derepression effect of *hsfb2b* is stronger at short-term cold and 40VT1 (1 d at room temperature after returning from 40 days of vernalization) than during vernalization (Fig. 2e and f).

Effects of vernalization on HsfB2b

Hsfb2b expression is well-known to be induced by heat shock treatment to suppress hyperactivated heat shock-responsive genes[20]. However, the effect on *Hsfb2b* is unknown. During vernalization, the levels of *Hsfb2b* did not change significantly, despite a slight increase when returned to room temperature (Fig. 3a). We also checked HsfB2b protein levels using *pHsfB2b::HsfB2b-eGFP* transgenic lines during vernalization (Fig. 3b and Suppl Fig. S4). However, the protein levels were not found to be significantly affected by vernalization. Nonetheless, vernalization caused retarded migration of HsfB2b-eGFP proteins on polyacrylamide gel from the initial phase, suggesting that HsfB2b undergoes post-translational modifications, such as phosphorylation by cold. After returning to room temperature, such modifications may have been rapidly erased as 40VT1 displayed the same protein pattern as NV.

As the cellular localisation of other Hsf is changed by post-translational modification[25], we checked whether cold treatment can change that of HsfB2b (Fig. 3c and d). Using the *pHsfB2b::HsfB2b-eGFP* transgenic lines, we observed the root tissue before and after 5 days of cold. In both cases, GFP signals were observed in the nucleus, indicating that neither cold treatment nor protein modification altered the cellular localisation of HsfB2b. This result is consistent with the fact that the protein sequence of HsfB2b has a nuclear localisation signal (NLS), but lacks a nuclear export signal (NES) motif[16]. Taken together, *HsfB2b* is neither transcriptionally induced nor is the subcellular localisation of the proteins altered by vernalization.

VIN3 expression is reported to show a circadian rhythm and *HsfB2b* acts as a negative regulator of the circadian clock regulator, *PSEUDO RESPONSE REGULATOR7* (*PRR7)*[22,23,26]. Thus, we verified whether the *VIN3* rhythm was affected by *hsfb2b* during cold treatment. Although the amplitude of the circadian rhythm was increased by *hsfb2b* mutation due to the increase in *VIN3* level, the rhythmic pattern was not significantly different (Fig. 3e and f). Therefore, *HsfB2b* seems to constitutively repress *VIN3,* and this repression is independent of the *HsfB2b*-regulated circadian clock.

HsfB2b directly regulates *VIN3* repression

Heat shock transcription factors regulate a variety of genes by directly binding to the HSE in the promoters[13,14]. Consistently, HSE was detected near the *VIN3* promoter, approximately 40 bp downstream of the transcription start site (Fig. 4a). In addition, the HSE was highly conserved among the *VIN3* orthologs from Brassicaceae species (*Arabidopsis thaliana*, *Arabidopsis lyrata, Boechera stricta*, and *Capsella rubella*) (Supplementary Fig. S2). Therefore, we determined whether HsfB2b directly binds to the *VIN3* promoter. In *silico* analyses using the DNA affinity purification (DAP)-seq database[27] showed that several heat shock transcription factors bind to the 5'-UTR of *VIN3*, where HSE is located (Supplementary Fig. S3).

We proceeded to assess whether HsfB2b bound to HSE_{VIN3} using yeast one-hybrid assay (Fig. 4b). Among the nine Hsf proteins analysed, HsfA1, HsfA6a, HsfB1, HsfB2b, and HsfC1 were found to interact with HSE_{VIN3}. To confirm *in planta* binding, we also performed chromatin immunoprecipitation-qPCR using transgenic *pHsfB2B::HsfB2b-eGFP,* grown under long days without cold treatment (Fig. 4a and c). HsfB2b-eGFP proteins were found to be enriched in the promoter region near the HSE location, even without cold treatment. Taken together, these data strongly support the hypothesis that HsfB2b directly regulates *VIN3* repression.

hsfb2b mutation does not change vernalization response under normal condition

To analyse the effect of *hsfb2b* on vernalization response, the *hsfb2b* mutation was introduced into *FRI* Col, a vernalization-sensitive line, by genetic cross[28]. As shown in Fig. 5, the *flowering time of hsfb2b FRI was similar to* that *of FRI* Col, although *VIN3* levels were higher in *hsfb2b FRI* than in *FRI* Col throughout the vernalization time course (Fig. 5a-c). Consistently, the *FLC* levels were not significantly different between the two genotypes throughout vernalization (Fig. 5d). Thus, the increased levels of *VIN3* in *hsfb2b* may not alter the vernalization response under normal growth conditions. Such findings suggests that *VIN3* levels in the *FRI* Col are sufficient for a proper vernalization response.

HsfB2b overexpression leads to hyposensitive response to vernalization

In our complementation analysis, all *HsfB2b* transgenic lines displayed overexpression of *HsfB2b*, although the transgenes were driven by the endogenous promoter. Thus, we analysed the vernalization response in *HsfB2b* overexpressing lines. The *hov1* mutant, containing a nonsense mutation in the first exon of *HsfB2b*, showed approximately 3-fold higher *HsfB2b* levels than Col-0, which might be due to the negative feedback regulation (Fig. 6a). When the transgenes, *pHsfB2b::HsfB2b-myc* or *pHsfB2b::HsfB2b-eGFP*, were introduced into the *hov1* background, the *HsfB2b* levels were increased by 20–50-fold relative to that of Col-0, indicating that the transgenic lines were *HsfB2b* overexpressors (Fig. 6a). The GUS and endogenous *VIN3* levels among the parental lines (*-0.2kb pVIN3_U_I::GUS*), *hov1* (in *-0.2kb pVIN3_U_I::GUS* background), and *pHsfB2b::HsfB2b-myc*

hov1 (Fig. 6b and 6c) were subsequently compared after 40 days of vernalization. The overexpression of *HsfB2b* was found to markedly reduce *VIN3* levels after 40 days of vernalization. Such finding is consistent with the hypothesis that HsfB2b represses *VIN3* transcription.

We proceeded to verify whether *HsfB2b* overexpression caused any changes in the vernalization response. Briefly, we introduced *pHsfB2b::HsfB2b-eGFP* into *FRI* Col by transformation. As expected, all 10 transgenic lines showed overexpression of *HsfB2b* (3~10 folds) based on the level after 20 days of vernalization (20V) (Fig. 6d). In these transgenic lines, *VIN3* levels were lower than those in both *FRI* Col and *hsfb2b FRI* after 20V, which supports the hypothesis that *HsfB2b* overexpression causes the repression of *VIN3* in *FRI* Col plants (Fig. 6d). Consistent with the fact that *VIN3* is required for the suppression of *FLC*[8], the *FLC* levels in *pHsfB2b::HsfB2b-eGFP FRI* lines were slightly higher than those in *FRI* Col after 20V (Fig. 6d). Finally, the flowering time of the transgenic lines, *pHsfB2b::HsfB2b-eGFP FRI,* was less accelerated than that of both *FRI* Col and *hsfb2b FRI* by 20V (Fig. 6e and f). Therefore, in contrast to *hsfb2b* mutation, *HsfB2b* overexpression causes defects in the vernalization response under normal growth conditions.

Discussion

VIN3 is required for proper vernalization in *Arabidopsis*, particularly winter annuals. However, the molecular mechanism by which *VIN3* is finely regulated has not been fully elucidated. In this study, we isolated a mutant, *hov1,* that showed hyperactivation of *VIN3*. By map-based cloning combined with whole-genome resequencing, *HsfB2b* was defined as the causative gene for *VIN3* derepression in *hov1*. Interestingly, *hsfb2b* exhibited higher *VIN3* levels under all conditions, including before and after vernalization. Therefore, *HsfB2b* might act as a general repressor of *VIN3*, regardless of cold treatment. Nonetheless, the intensity of the derepression in *hsfb2b* was strongest at the initial stages of cold treatment and stronger at the phase of return to room temperature after 40V than during vernalization. *HsfB2b* might act in a fine-tuning mechanism, suppressing precocious *VIN3* activation during the fall when temperature drops abruptly and rapidly suppressing *VIN3* levels after spring.

Higher *VIN3* levels in *hsfb2b* failed to show a higher vernalization response in the late-flowering *FRI* Col background. This result is consistent with that of previous studies where ectopic expression of *VIN3* was not found to alter the vernalization response, despite complementing *vin3* mutation[29,30]. In contrast, the lower *VIN3* levels in the *HsfB2b* overexpression lines caused a weak vernalization response in both the acceleration of flowering time and *FLC* suppression by 20V. This result is also consistent with the fact that the *vin3* mutation causes failure of the vernalization response[7,8]. Finally, HsfB2b was found to directly repress *VIN3,* a key factor in the vernalization process, by binding to the HSE on the 5'-UTR of *VIN3* (Fig. 5). Of note, the HSE_{VIN3} sequences on the 5'-UTR of the *VIN3* orthologues are highly conserved*,* whereas other regions of the 5'-UTR are relatively diversified among Brassicaceae species. As vernalization responses have been observed throughout Brassicaceae, conservation of such cis-elements suggests that *VIN3* regulation by Hsfs may also be conserved across the Brassicaceae family[31-33].

In *Arabidopsis,* Hsfs have been reported to regulate diverse stress responses, including responses to both biotic and abiotic stresses, such as bacterial infection, fungal infection, and heat and drought stresses[34-37]. During such responses, both class A and class B Hsfs are incorporated into complex and multi-layered regulatory systems, and different combinations of Hsfs seem to act on each stress response[18]. Here, *HsfB1* was not functionally redundant with *HsfB2b* for *VIN3* regulation (Fig. 2). However, several Hsfs, besides HsfB2b, including HsfB1, bound to the HSE_{VIN3} elements present in the 5'-UTR of *VIN3* in the yeast one-hybrid assay (Fig. 5). Therefore, other Hsfs, recognising $HSE_{VIN3,}$ may regulate *VIN3* transcription in response to other stresses, such as low oxygen conditions at which *VIN3* is induced[38]. This notion is consistent with the finding that Hsfs are required for a broad range of stress responses[13,17,18]. It would be interesting to determine whether VIN3 acts as a hub for the stress responses mediated by Hsfs.

Plants perceive winter cold as a signal for vernalization, but simultaneously perceive it as long-term cold stress. In *Arabidopsis*, several HSPs and factors are strongly induced by cold stress, and the roles of both HSPs and Hsfs in the cellular response to cold stress have been reported previously[13,39-41]. The HsfB2b protein displayed retarded migration on polyacrylamide gels during vernalization. Such cold-induced post-translational modifications indicate that *HsfB2b* is involved in a subset of cold signal transduction (Fig. 3b). Moreover, the transcript level of *HsfB2b* was slightly elevated after returning from cold temperatures to warm temperatures (Fig. 3a). Such

observations may indicate that *HsfB2b* is required for sensing temperature changes, which are inevitable during vernalization. Thus, *VIN3* regulation by *HsfB2b* may have evolved from a mechanism that senses cold stress.

The circadian clock was previously demonstrated to be involved in the regulation of *VIN3*, and components of the circadian clock, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *LATE ELONGATED HYPOCOTYL* (*LHY)*, directly regulate the diurnal rhythm of *VIN3* during vernalization[23,26]. One of the circadian clock regulators, *PRR7*, has also been reported to be a transcription factor repressed by *HsfB2b*[22], which is required for proper abiotic stress responses. However, our data indicate that *HsfB2b* is not involved in regulating the diurnal rhythm of *VIN3* under cold treatment, despite affecting the amplitude (Fig. 3e and f). As the circadian clock has rhythmic robustness due to multiple feedback loops consisting of diverse transcription factors, the defect in clock regulation by *hsfb2b* seems to be minor for the *VIN3* rhythm[42].

Under natural conditions, where environmental changes markedly occur, plants must avoid and distinguish between uncertain signals. For vernalization, plants must distinguish transient changes in temperature from winter cold. For example, plants often experience a sudden cold during late fall or a sudden warmth in early spring. Thus, plants must have an elaborate mechanism to regulate *VIN3* expression in response to ever-changing environmental conditions. Consistently, *VIN3* has been demonstrated to display dynamic expression patterns depending on fluctuating temperature[26]. For such elaborate regulation of *VIN3*, *HsfB2b* may provide a fine-tuning mechanism to prevent unintentional flowering from sudden cold.

Materials and Methods

Plant Materials and Growth Conditions

All *Arabidopsis thaliana* lines used were in the Columbia (Col-0) background except L*er* ecotype used to generate mapping population for map-based gene cloning. The wild-type, Col:*FRI*^{Sf2} (*FRI* Col) have been previously described[28]. *hsfb1, hsfb2b*, and *hsfb1 hsfb2b* mutants have been previously described[20].

To produce *pHsfB2b::HsfB2b-eGFP* construct, the genomic sequences including 2,624bp upstream of the promoter and the whole coding sequence of *HsfB2b* were amplified by polymerase chain reaction. The fragment was cloned into *pCR2.1-TOPO* vector, then fused in-frame to *pCAMBIA1300* vector containing eGFP. The construct was transformed into the *hov1* mutant. To produce *pHsfB2b::Hsfb2b-myc*, the 3 kb *HsfB2b* promoter and the HsfB2b-coding sequence were amplified and fused in-frame to *pPZP221* vector containing 4xmyc (EQKLISEEDL). The construct was transformed into the indicated lines using Agrobacterium (*Agrobacterium tumefaciens*)-mediated *Arabidopsis* floral dip method[43].

The plants were grown under 16 h/8 h light/dark cycle (long day) or 8 h/16 h light/dark cycle (short day) (22°C/20°C) in a controlled growth room with cool white fluorescent lights (125 μmol m-2 sec-1). Vernalization treatments were done as previously described (Kim and Sung, 2013). Nonvernalized seedlings were grown for 11 d. For 10V, 20V, 40V treatments, seedlings were grown for 10, 9, 7 d under short days respectively after germination, then transferred to vernalization chamber at 4°C. After vernalization treatment, seedlings were collected or transplanted to the soil. Flowering time was measured by counting the number of rosette leaves when bolting using at least 20 plants.

EMS mutagenesis and positional cloning

EMS mutagenesis was performed as previously described[44]. For the positional cloning of the causative gene of *hov1*, F2 progenies were obtained by crossing *hov1* to L*er*. Mapping procedure was followed using 135 GUS-hypersensitive F2 plants and molecular makers described as previous reports[45,46]. After rough mapping, the genomes of *hov1* and the parental -*0.2kb pVIN3_U_I::GUS* were sequenced and compared by illumina Hiseq2000 platform (illumina) sequencing to find mutant-specific SNPs in *hov1* using BGI services.

Histochemical GUS Staining

GUS staining was done following the standard methods that have been previously described (Shin et al., 2018). Photographs were taken with a USB digital-microscope Dimis-M (Siwon Optical Technology, South Korea).

Quantitative PCR

For real-time quantitative PCR, total RNA was isolated using TRIzol solution (Sigma). Four micrograms of total RNA were treated with recombinant DNase I (TaKaRa, 2270A) to eliminate genomic DNA. cDNA was generated using the RNA with reverse transcriptase (Thermo scientific, EP0441) and oligo(dT). Quantitative PCR was performed using the 2x SYBR Green SuperMix (Bio-Rad 170-8882) and monitored by the CFX96 real-time PCR detection system. The relative transcript level of each gene was determined by normalization of the resulting

expression levels compared to that of UBC. The primer sequences used in real-time RT-PCR analyses were shown in Supplementary Table S1.

Promoter Analysis

The promoter sequences from plant species were downloaded from GBrowse at Phytozome (www.phytozome.net). The following *VIN3* loci (At5g57380) were identified using BLAST Search: *Arabidopsis lyrata* (AL8G33360), *Boechera stricta* (Bostr.26833s0518) and *Capsella rubella* (Carub.0008s1790). The sequences were processed and aligned in T-coffee (tcoffee.crg.eu)

Yeast one-hybrid assay

Yeast one-hybrid assay was performed following the previously described method with some modifications[23]. For the reporter constructs used in the Y1H analysis, four tandem repeats containing HSE_{VIN3} (5'-TTAGAAACATCTAGAAAAAACAAA-3') were cloned into the *pHisi* vector. For the effector, the coding sequences of *HsfA1a, HsfA2, HsfA4a, HsfA6a, HsfA8, HsfB1, HsfB2b* and *HsfC1* were cloned in-frame with the sequences of the GAL4 activation domain into *pGADT7*. The Y1H assay was performed following the manufacturer's instructions. In brief, the reporter construct and effector construct were transformed into yeast strain YM4271. The yeast cells were spotted on synthetic define (SD) medium lacking Leu, Ura, and His, with or without 5-mM 3-amino-1,2,4-triazole (3-AT).

Chromatin Immunoprecipitation

Approximately 4 g of whole *Arabidopsis* seedlings were collected and cross-linked using 1% (v/v) formaldehyde for 10 min and quenched by 0.125 M glycine for 5 min under vacuum. Seedlings were rinsed with distilled water, frozen in liquid nitrogen, and grounded to fine powder. The powder was resuspended in Nuclei Isolation Buffer [1 M hexylene glycol, 20 mM PIPES-KOH (pH 7.6), 10 mM $MgCl_2$, 15 mM NaCl, 1 mm EGTA, 1 mM PMSF, complete protease inhibitor mixture tablets (Roche)], and *Arabidopsis* nuclei were isolated by centrifugation, lysed by Nuclei Lysis Buffer [50 mM TRIS-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% SDS], and sonicated using a Branson sonifier to shear the DNA. Sheared chromatin solution was diluted 10-fold with a ChIP Dilution Buffer [50 mM TRIS-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA]. The beads, chromatins and GFP-Trap A beads (gta-20, ChromoTek, Planegg, Germany) or Binding control agarose (bab-20) were mixed and incubated for overnight at 4°C. Be5ads were washed with ChIP dilution buffer for 4 times and DNA extraction was performed using Chelex 100 resin following the manufacturer's instruction. qPCR analysis was performed using 1% input and immunoprecipitated DNA.

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for the genes discussed in this paper are as follows: *VIN3* (At5g57380), *FLC* (At5g10140), *FRI* (At4g00650), *HsfA1a* (At4g17750), *HsfA2* (At2g26150), *HsfA3* (At5g03720), *HsfA4a* (At4g18880), *HsfA6a* (At5g43840), *HsfA8* (At1g67970), *HsfB1* (At4g36990), *HsfB2a* (At5g62020), *HsfB2b* (At4g11660), *HsfC1* (At3g24520), *CCA1* (At2g46830), *LHY* (At1g01060), and *PP2A* (At1g13320).

Statements

All the plant materials and methods used in the current study were carried out following relevant institutional, national, and international guidelines and legislation.

References

1 Chouard, P. Vernalization and its Relations to Dormancy. *Annual Review of Plant Physiology* **11**, 191-238, doi:10.1146/annurev.pp.11.060160.001203 (1960).

2 Michaels, S. D. & Amasino, R. M. Memories of winter: vernalization and the competence to flower. *Plant, Cell & Environment* **23**, 1145-1153, doi:https://doi.org/10.1046/j.1365-3040.2000.00643.x (2000).

3 Sheldon, C. C. *et al.* The FLF MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation. *The Plant Cell* **11**, 445-458, doi:10.1105/tpc.11.3.445 (1999).

4 Michaels, S. D. & Amasino, R. M. FLOWERING LOCUS C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering. *The Plant Cell* **11**, 949-956, doi:10.1105/tpc.11.5.949 (1999).

5 De Lucia, F., Crevillen, P., Jones, A. M. E., Greb, T. & Dean, C. A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of FLC during vernalization. *Proceedings of the National Academy of Sciences* **105**, 16831, doi:10.1073/pnas.0808687105 (2008).

6 Wood, C. C. *et al.* The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. *Proceedings of the National Academy of Sciences* **103**, 14631-14636, doi:10.1073/pnas.0606385103 (2006).

7 Yang, H. *et al.* Distinct phases of Polycomb silencing to hold epigenetic memory of cold in Arabidopsis. *Science* **357**, 1142-1145, doi:10.1126/science.aan1121 (2017).

8 Sung, S. & Amasino, R. M. Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature* **427**, 159-164, doi:10.1038/nature02195 (2004).

9 Richter, K., Haslbeck, M. & Buchner, J. The heat shock response: life on the verge of death. *Mol Cell* **40**, 253-266, doi:10.1016/j.molcel.2010.10.006 (2010).

10 Feder, M. E. & Hofmann, G. E. HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. *Annual Review of Physiology* **61**, 243-282, doi:10.1146/annurev.physiol.61.1.243 (1999).

11 Wu, C. Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* **11**, 441-469, doi:10.1146/annurev.cb.11.110195.002301 (1995).

12 Enoki, Y. & Sakurai, H. Diversity in DNA recognition by heat shock transcription factors (HSFs) from model organisms. *FEBS Letters* **585**, 1293-1298, doi:https://doi.org/10.1016/j.febslet.2011.04.014 (2011).

13 Busch, W., Wunderlich, M. & Schöffl, F. Identification of novel heat shock factor-dependent genes and biochemical pathways in Arabidopsis thaliana. *The Plant Journal* **41**, 1-14, doi:https://doi.org/10.1111/j.1365-313X.2004.02272.x (2005).

14 Amin, J., Ananthan, J. & Voellmy, R. Key features of heat shock regulatory elements. *Mol Cell Biol* **8**, 3761-3769, doi:10.1128/mcb.8.9.3761-3769.1988 (1988).

15 Nover, L. *et al.* The Hsf world: classification and properties of plant heat stress transcription factors. *Cell*

Stress Chaperones **1**, 215-223, doi:10.1379/1466-1268(1996)001 (1996).

16 Nover, L. *et al.* Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? *Cell Stress Chaperones* **6**, 177-189, doi:10.1379/1466-1268(2001)006 (2001).

17 von Koskull-Döring, P., Scharf, K.-D. & Nover, L. The diversity of plant heat stress transcription factors. *Trends in Plant Science* **12**, 452-457, doi:https://doi.org/10.1016/j.tplants.2007.08.014 (2007).

18 Andrási, N., Pettkó-Szandtner, A. & Szabados, L. Diversity of plant heat shock factors: regulation, interactions, and functions. *Journal of Experimental Botany* **72**, 1558-1575, doi:10.1093/jxb/eraa576 (2021).

19 Ikeda, M. & Ohme-Takagi, M. A novel group of transcriptional repressors in Arabidopsis. *Plant Cell Physiol* **50**, 970-975, doi:10.1093/pcp/pcp048 (2009).

20 Ikeda, M., Mitsuda, N. & Ohme-Takagi, M. Arabidopsis HsfB1 and HsfB2b Act as Repressors of the Expression of Heat-Inducible Hsfs But Positively Regulate the Acquired Thermotolerance. *Plant physiology* **157**, 1243-1254, doi:10.1104/pp.111.179036 (2011).

21 Kumar, M. *et al.* Heat Shock Factors HsfB1 and HsfB2b Are Involved in the Regulation of Pdf1.2 Expression and Pathogen Resistance in Arabidopsis. *Molecular Plant* **2**, 152-165, doi:https://doi.org/10.1093/mp/ssn095 (2009).

22 Kolmos, E., Chow, B. Y., Pruneda-Paz, J. L. & Kay, S. A. HsfB2b-mediated repression of PRR7 directs abiotic stress responses of the circadian clock. *Proceedings of the National Academy of Sciences* **111**, 16172, doi:10.1073/pnas.1418483111 (2014).

23 Kyung, J. *et al.* The two clock proteins CCA1 and LHY activate VIN3 transcription during vernalization through the vernalization-responsive cis-element. *The Plant Cell*, koab304, doi:10.1093/plcell/koab304 (2021).

24 Bond, D. M., Wilson, I. W., Dennis, E. S., Pogson, B. J. & Jean Finnegan, E. VERNALIZATION INSENSITIVE 3 (VIN3) is required for the response of Arabidopsis thaliana seedlings exposed to low oxygen conditions. *The Plant Journal* **59**, 576-587, doi:https://doi.org/10.1111/j.1365-313X.2009.03891.x (2009).

25 Evrard, A. *et al.* Regulation of the heat stress response in Arabidopsis by MPK6-targeted phosphorylation of the heat stress factor HsfA2. *PeerJ* **1**, e59, doi:10.7717/peerj.59 (2013).

26 Hepworth, J. *et al.* Absence of warmth permits epigenetic memory of winter in Arabidopsis. *Nature Communications* **9**, 639, doi:10.1038/s41467-018-03065-7 (2018).

27 O'Malley, R. C. *et al.* Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. *Cell* **165**, 1280-1292, doi:10.1016/j.cell.2016.04.038 (2016).

28 Lee, I., Michaels, S. D., Masshardt, A. S. & Amasino, R. M. The late-flowering phenotype of FRIGIDA and mutations in LUMINIDEPENDENS is suppressed in the Landsberg erecta strain of Arabidopsis. *The Plant Journal* **6**, 903-909, doi:https://doi.org/10.1046/j.1365-313X.1994.6060903.x (1994).

29 Lee, J., Yun, J.-Y., Zhao, W., Shen, W.-H. & Amasino, R. A methyltransferase required for proper timing of the vernalization response in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **112**, doi:10.1073/pnas.1423585112 (2015).

30 Kim, D.-H. & Sung, S. The Binding Specificity of the PHD-Finger Domain of VIN3 Moderates

Vernalization Response. *Plant Physiology* **173**, 1258-1268, doi:10.1104/pp.16.01320 (2017).

31 Kuittinen, H., Niittyvuopio, A., Rinne, P. & Savolainen, O. Natural Variation in Arabidopsis lyrata Vernalization Requirement Conferred by a FRIGIDA Indel Polymorphism. *Molecular Biology and Evolution* **25**, 319-329, doi:10.1093/molbev/msm257 (2008).

32 Anderson, J. T., Lee, C.-R. & Mitchell-Olds, T. Life-history QTLS and natural selection on flowering time in Boechera stricta, a perennial relative of Arabidopsis. *Evolution* **65**, 771-787, doi:10.1111/j.1558-5646.2010.01175.x (2011).

33 Guo, Y.-L., Todesco, M., Hagmann, J., Das, S. & Weigel, D. Independent FLC Mutations as Causes of Flowering-Time Variation in Arabidopsis thaliana and Capsella rubella. *Genetics* **192**, 729-739, doi:10.1534/genetics.112.143958 (2012).

34 Yoshida, T. *et al.* Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol Genet Genomics* **286**, 321-332, doi:10.1007/s00438-011-0647-7 (2011).

35 Bechtold, U. *et al.* Arabidopsis HEAT SHOCK TRANSCRIPTION FACTORA1b overexpression enhances water productivity, resistance to drought, and infection. *J Exp Bot* **64**, 3467-3481, doi:10.1093/jxb/ert185 (2013).

36 Nishizawa, A. *et al.* Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *The Plant Journal* **48**, 535-547, doi:https://doi.org/10.1111/j.1365-313X.2006.02889.x (2006).

37 Ogawa, D., Yamaguchi, K. & Nishiuchi, T. High-level overexpression of the Arabidopsis HsfA2 gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *Journal of Experimental Botany* **58**, 3373-3383, doi:10.1093/jxb/erm184 (2007).

38 Banti, V., Mafessoni, F., Loreti, E., Alpi, A. & Perata, P. The heat-inducible transcription factor HsfA2 enhances anoxia tolerance in Arabidopsis. *Plant physiology* **152**, 1471-1483, doi:10.1104/pp.109.149815 (2010).

39 Sabehat, A., Lurie, S. & Weiss, D. Expression of small heat-shock proteins at low temperatures. A possible role in protecting against chilling injuries. *Plant Physiol* **117**, 651-658, doi:10.1104/pp.117.2.651 (1998).

40 Ul Haq, S. *et al.* Heat Shock Proteins: Dynamic Biomolecules to Counter Plant Biotic and Abiotic Stresses. *Int J Mol Sci* **20**, doi:10.3390/ijms20215321 (2019).

41 Swindell, W. R., Huebner, M. & Weber, A. P. Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics* **8**, 125-125, doi:10.1186/1471-2164-8-125 (2007).

42 Shalit-Kaneh, A., Kumimoto Roderick, W., Filkov, V. & Harmer Stacey, L. Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. *Proceedings of the National Academy of Sciences* **115**, 7147-7152, doi:10.1073/pnas.1805524115 (2018).

43 Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-743, doi:10.1046/j.1365-313x.1998.00343.x (1998).

44 Kim, Y., Schumaker, K. S. & Zhu, J. K. EMS mutagenesis of Arabidopsis. *Methods Mol Biol* **323**, 101-103, doi:10.1385/1-59745-003-0:101 (2006).

45 Lukowitz, W., Gillmor, C. S. & Scheible, W.-R. d. Positional Cloning in Arabidopsis. Why It Feels Good to Have a Genome Initiative Working for You1. *Plant Physiology* **123**, 795-806, doi:10.1104/pp.123.3.795 (2000).

46 Hou, X. *et al.* A platform of high-density INDEL/CAPS markers for map-based cloning in Arabidopsis. *Plant J* **63**, 880-888, doi:10.1111/j.1365-313X.2010.04277.x (2010).

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Contributions

G. J., J. S. and I. L. conceived and designed the experiments; J. S. and G. J. performed the experiments. G. J., J. S. and I. L. performed data analysis. G. J. and I. L. prepared the manuscript. All authors have contributed in writing the manuscript and have read and approved the final version.

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Competing interests

The authors declare no competing interests.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Supplementary information

Supplementary Table S1. Primers names and their sequences used for vector construction and quantitative PCR in this study

Supplementary Figure S1. Map-based cloning reveals the causative gene of the *hov1* mutation

Supplementary Figure S2. HSE_{VIN3} is conserved among *Arabidopsis* relatives

Supplementary Figure S3. DNA Affinity Purification (DAP)-seq reveals *in vitro* binding of HsfB2b to *VIN3*

Supplementary Figure S4. Original images of representative immunoblot

Figures

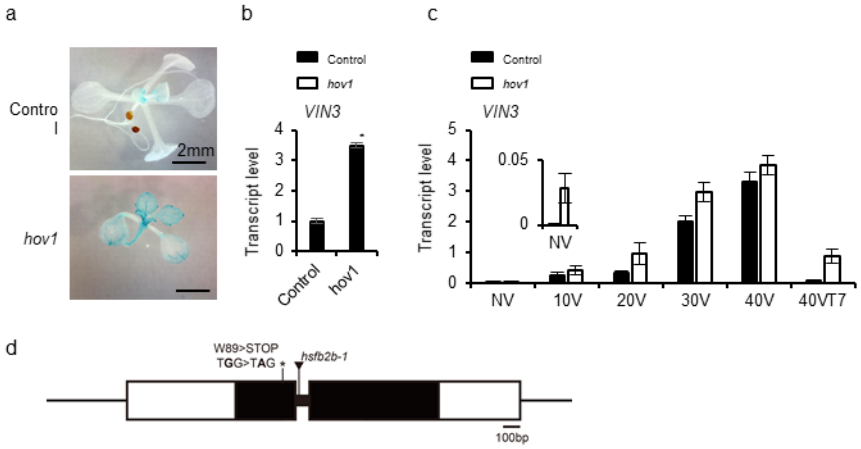


Figure 1. Isolation and characterization of the vernalization hypersensitive mutant, *hov1*

(a-b) Characterization of the *hov1* mutant. Seedlings of the control (*-0.2kb pVIN3_U_I::GUS*) and *hov1* were grown at room temperature for 10 days and analysed after 3 days of cold treatment. (a) Images of representative seedlings after GUS staining. (Scale bars, 2 mm) (b) Endogenous *VIN3* transcript levels in control and *hov1*. Data are presented as mean ± SEM of three biological replicates. Asterisks indicate significant difference compared with the control (Student's t-test; ***P<0.001).

(c) Time-course analysis of *VIN3* levels during vernalization. NV, non-vernalized; 10V, 20V, 30V, and 40V, 10 d, 20 d, 30 d, and 40 d, respectively; 40VT7, 7 d grown at room temperature after 40V. Data are presented as mean ± SEM of three biological replicates. The inset in (c) is enlarged for NV.

(d) Schematic structure of the *HsfB2b* gene. Black bars indicate exons, and white boxes and lines represent untranslated regions and introns, respectively. The mutations that occurred in the two alleles are shown: T-DNA insertion as a triangle and point mutation as an asterisk.

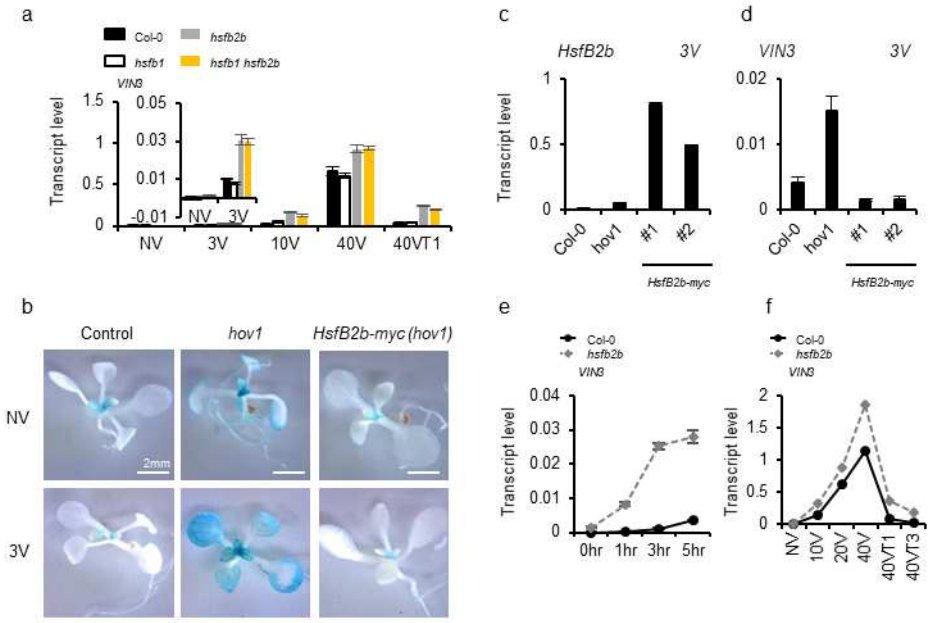


Figure 2. HsfB2b acts as a transcriptional repressor of *VIN3*

(a) *HsfB1* is not functionally redundant with *HsfB2b* in *VIN3* regulation. *VIN3* transcript levels in Col-0, *hsfb1, hsfb2b,*and *hsfb1 hsfb2b* during vernalization were determined using RT-qPCR. NV, non-vernalized; 3V, 10V, 40V, 3, 10, and 40 d vernalized; 40VT1, 1 d growth at room temperature after 40V. Transcript levels were normalized to those of *PP2A*. Data are presented as mean ± SEM of three biological replicates. The inset in (a) is enlarged for the NV at 3V.

(b-d) Complementation of *hov1* with *pHsfB2b::HsfB2b-myc*. (b) GUS staining of NV or 3V seedlings of the parental line, *hov1*, and *pHsfB2b::HsfB2b-myc hov1* transgenic line. Images of representative seedlings after GUS staining (c) *HsfB2b* or (d) *VIN3* transcript levels in 3V Col-0, *hov1*, and two representative transgenic lines of *pHsfB2b::HsfB2b-myc hov1*. Transcript levels were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates.

(e-f) Effects of *hsfb2b* mutation on *VIN3* levels. *VIN3* levels in Col-0 and *hsfb2b*, as determined by RT-qPCR after (e) short-term cold treatment (0, 1, 3, and 5 h) or (f) long-term vernalization (NV, 10V, 20V, 40V, 40VT1, 40VT3). Transcript levels were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates.

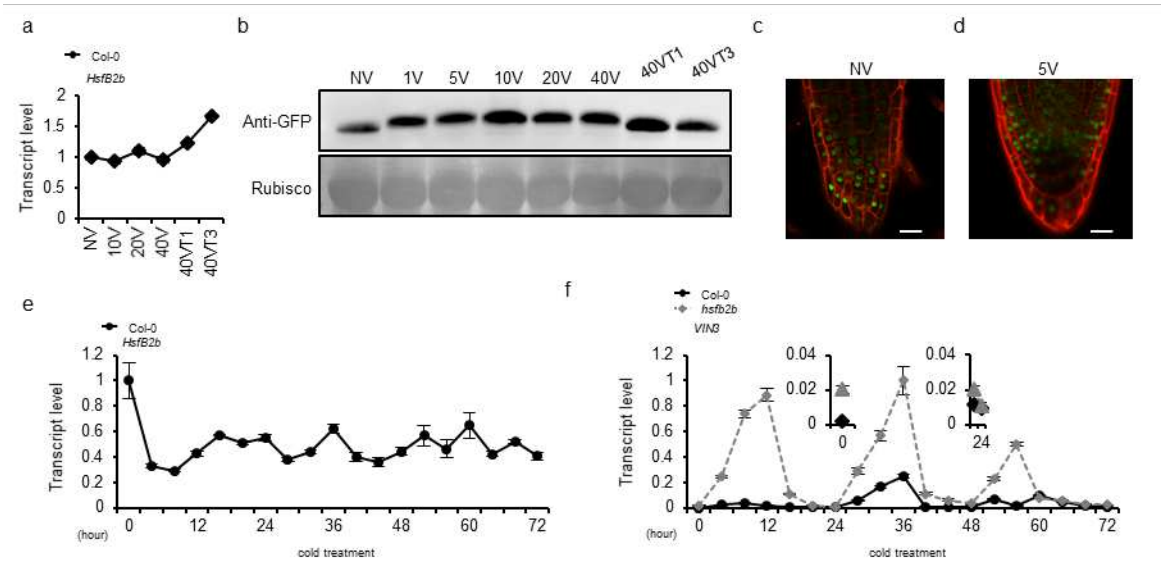


Figure 3. Characterization of *Hsfb2b* during cold and vernalization treatment

(a) Effect of vernalization on the transcript levels of *HsfB2b*. Col-0 seedlings were vernalized before total RNA extraction for RT-PCR analysis. Expression levels were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates. (b) Immunoblot analysis of the HsfB2b-eGFP protein extracted from vernalized seedlings of *pHsfB2b::HsfB2b-eGFP*. Rubisco was used as a loading control. NV, non-vernalized; 1V, 5V, 10V, 20V and 40V, 1 d, 5 d, 10 d, 20 d, and 40 d, respectively; 40VT1, 40VT3, 1 d, and 3 d, grown at room temperature after 40V, respectively. (c-d) Confocal images of roots from NV or 5V plants expressing *pHsfB2b::HsfB2b-eGFP*. (Scale bars, 20 μm) Five days-old *Arabidopsis* seedlings, with or without 5 days of cold exposure, were harvested and counterstained with propidium iodide. (e) Effect of the early phase of vernalization on the rhythmic expression of *HsfB2b* in Col-0. Expression levels were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates. (f) Effect of the *hsfb2b* mutation on the rhythmic expression of *VIN3*. *VIN3* levels during the early phase of vernalization were analysed using seedlings of Col-0 and *hsfb2b* collected at 4-h intervals over 72 h in LD at 4 °C. The x-axis indicates the exposure time to cold. Data are presented as mean ± SD of three technical replicates.

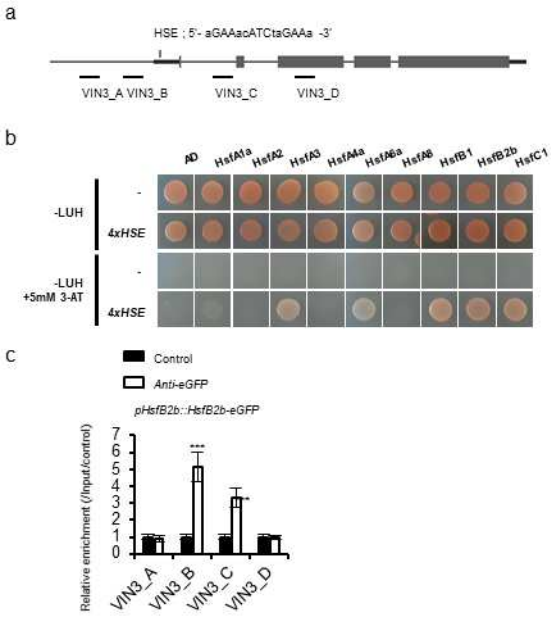


Figure 4. HsfB2b directly binds to the HSE on the *VIN3* locus.

(a) Schematic of the *VIN3* gene with description of the HSE_{VIN3} sequence and PCR amplicons. A-D, amplicons used for ChIP-qPCR. Black bars indicate untranslated region, and grey boxes and lines represent exons and introns, respectively.

(b) Yeast one-hybrid assay between heat shock factors and HSE_{VIN3} cis-element. As DNA baits, four tandem copies of the 24-bp sequences containing HSE_{VIN3} were inserted into the *pHisI* vector and used as the reporter construct. The CDS of HsfA1a, HsfA2, HsfA3, HsfA4a, HsfA6a, HsfA8, HsfB1, HsfB2b, and HsfC1 was cloned into *pGADT7* and used as an effector construct. GAL4 AD alone (AD) was used as the control. The effector and reporter constructs were co-transformed into the yeast strain, AH109. Representative growth status of yeast cells is shown on synthetic defined (SD)-LUH medium, with or without 5 mM 3-AT. LUH, SD medium without Leu, Ura, His; -LUH +5 mM 3-AT, SD medium without Leu, Ura, His but containing 5 mM 3-Amino-1,2,4-triazole.

(c) ChIP-qPCR showing the enrichment of HsfB2b-eGFP. Chromatin of the transgenic line expressing *pHsfB2b::HsfB2b-eGFP* was immunoprecipitated using control beads or GFP-trap beads. Histograms show mean values ± SEM (n=2 biological replicates, each biological replicate is an average value of three technical replicates) for enrichment calculated by percent input normalized against the control. Asterisks indicate significant differences compared with the control (Student's t-test; ***P<0.001).

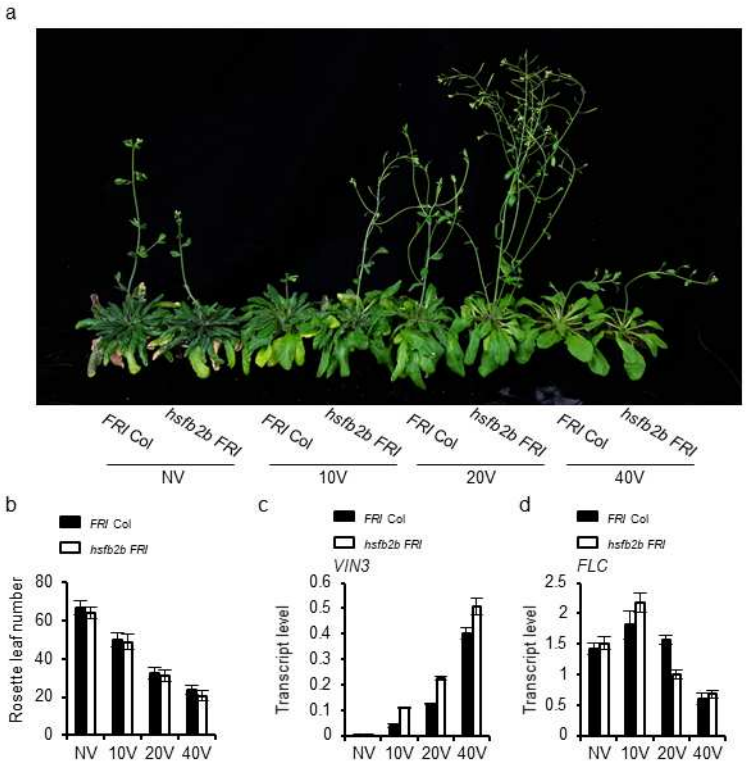
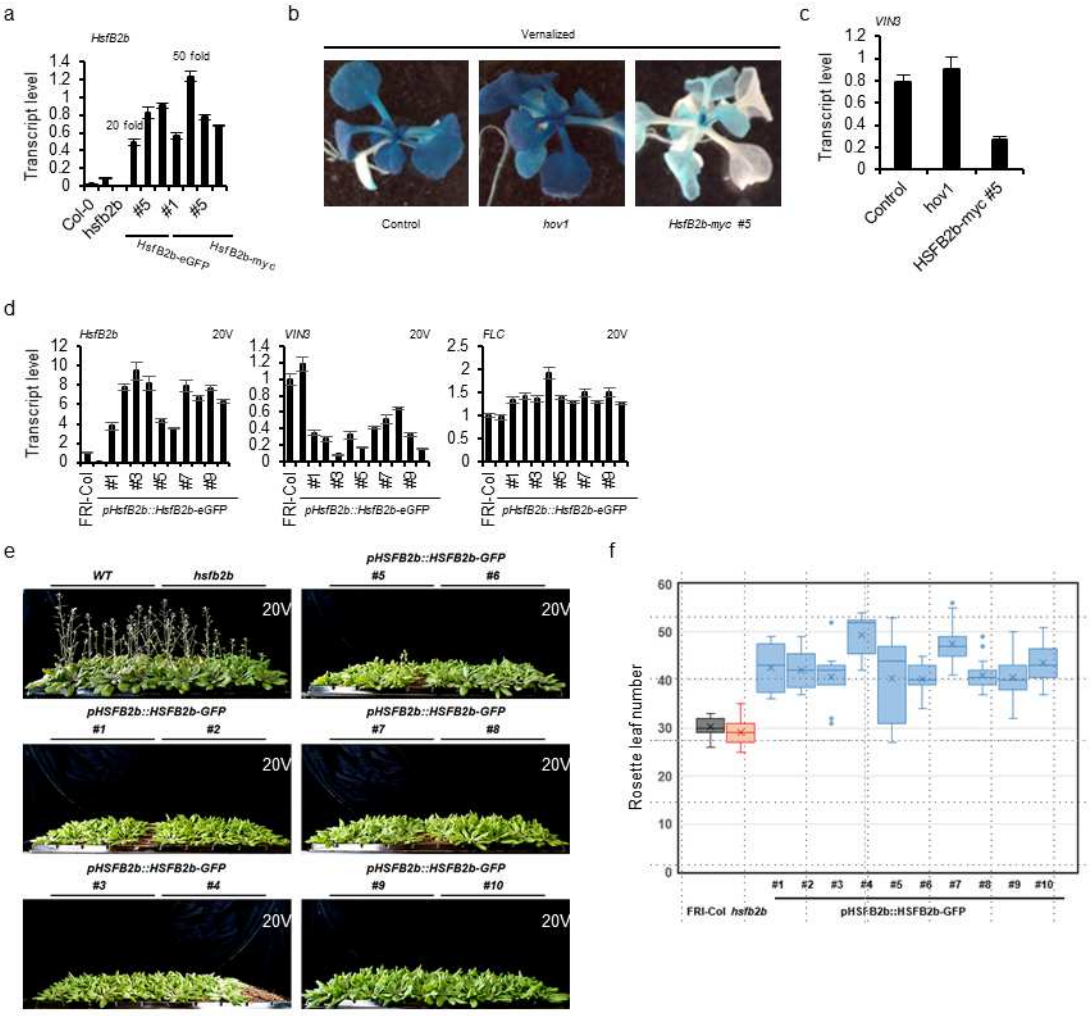


Figure 5. *hsfb2b* mutation does not change vernalization response under normal condition.

(a) Images of *FRI* Col and *hsfb2b FRI* without (NV) or with 10 d (10V), 20 d (20V), 40 d (40V) of vernalization.

(b) Flowering time of *FRI* Col and *hsfb2b FRI* after vernalization. Flowering time was measured as the number of primary rosette leaves formed when bolting. Data are presented as mean ± SD.

(c-d) *VIN3* or *FLC* transcript levels in *FRI* Col and *hsfb2b FRI* after vernalization treatment. The transcript levels were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates.



552 **Figure 6. *HsfB2b* overexpression leads to hyposensitive response to vernalization.**

553 (a) Comparison of the *HsfB2b* transcript levels in Col-0, *hov1*, *hsfb2b,* and the transgenic lines expressing
554 *pHsfB2b::HsfB2b-eGFP* or *pHsfB2b::HsfB2b-myc* in *hov1*. Transcript levels were normalized to those of *PP2A*.
555 Fold changes relative to Col-0 were marked for comparison. Data are presented as mean ± SD of three technical
556 replicates.

557 (b) GUS staining for 40V seedlings of the parental lines (*-0.2kb pVIN3_U_I::GUS*), *hov1*, and *pHsfB2b::HsfB2b-*
558 *myc hov1.* Images of representative seedlings after GUS staining are shown.

559 (c) *VIN3* levels in 40V seedlings of the parental line, *hov1*, and *pHsfB2b::HsfB2b-myc hov1.* Transcript levels
560 were normalized to those of *PP2A*. Data are presented as mean ± SD of three technical replicates.

561 (d) Transcript levels of *HsfB2b*, *VIN3*, and *FLC* in *FRI* Col, *hsfb2b FRI*, and transgenics expressing
562 *pHsfB2b::HsfB2b-eGFP* in *FRI* Col after 20V. Transcript levels were normalized to those of *PP2A*. Data are
563 presented as mean ± SD of three technical replicates.

564 (e) Photographs of WT (*FRI* Col), *hsfb2b FRI*, and transgenics expressing *pHsfB2b::HsfB2b-eGFP* in *FRI* Col
565 after 20V. Images captured when 20V WT and *hsfb2b* plants were fully flowered. (f) Flowering time is presented
566 as a box plot. The number of primary leaves when bolting was counted for flowering time. The centre lines indicate
567 the medians and × signs indicate the mean value. Box limits indicate the 25th and 75th percentiles.

Supplementary Files

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