A histone H4 gene prevents premature bolting by attenuating photoperiodic flowering genes under drought conditions in Chinese cabbage

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Abstract

Flowering is one of the most important traits in Chinese cabbage because premature flowering reduces yield and quality of the harvested products. Water deficit, caused by drought or other environmental conditions, can induce early flowering. Drought resistance involves global reprogramming of transcription, hormone signaling, and chromatin modification. How these regulatory responses are coordinated via the various pathways is largely unknown. We show that a histone H4 protein, BrHIS4.A04, physically interacts with a homeodomain protein BrVIN3.1 that was selected during the domestication of late-bolting Chinese cabbages. Over-expression of BrHIS4.A04 resulted in drought hypersensitivity and premature flowering under normal conditions but prevented premature bolting under drought conditions. We show that the expression of key ABA signaling genes (ABI1, MYC2, ABA1, and NCED3), and also photoperiodic flowering genes (GI, FT, and SOC1) was attenuated by BrHIS4.A04 under drought conditions. Furthermore, the level of H4-acetylation at these gene loci was hampered in BrHIS4.A04OE plants. BrHIS4.A04 prevents premature bolting by attenuating photoperiodic flowering genes under drought conditions through the ABA signaling pathway. Since BrHIS4.A04OE plants displayed no phenotypes related to vegetative or reproductive development under drought, our findings will contribute to fine-tuning of the flowering time in crops with no growth penalty through genetic engineering.

Introduction

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is the most important leafy crop in Asia; the annual production of Chinese cabbage in Asia is >50 million tons, which is approximately 70% of the world's output (Su et al., 2018). Flowering or bolting time is one of the most important traits in breeding Chinese cabbage because premature flowering reduces, or completely damages in some severe cases, the yield and quality of the harvested leafy products. With the requirements for a year-round vegetable supply and effective use of planting area, the demand for ecologically-targeted bolting-resistant varieties is strong and is increasing (Su et al., 2018).

Decades of physiological studies have shown that flowering is initiated in response to both environmental cues and endogenous pathways (Franks & Weis, 2008). In Arabidopsis, flowering time is mainly regulated through five pathways: vernalization, photoperiod, gibberellin, autonomous, and aging (Fornara, de Montaigu, & Coupland, 2010). Several abiotic stresses, such as drought and ambient temperature, can induce flowering, suggesting that plants can integrate the effects of abiotic stress with flowering signaling pathways (Andres & Coupland, 2012). Chinese cabbage is a high water-consuming crop, and drought stress, a very common

abiotic stress that is caused by water deficit, causes a series of physiological and molecular responses in plants. Water deficit during the growing season or in some area can lead to early bolting and insufficient vegetative growth, which ultimately affects the yield and quality of Chinese cabbage. From a broad perspective, crop drought sensitivity has been increased in the past two decades, and it is predicted that drought stress will cause severe problems in plant growth and crop production in more than 50% of agriculture lands by 2050 (Vurukonda, Vardharajula, Shrivastava, & Skz, 2015; Yang, Vanderbeld, Wan, & Huang, 2010). Environmental adaptation is important for optimal yield in the major crop plants. This includes adaptation of the reproductive system to the prevailing climatic conditions and an appropriate response to biotic and abiotic stresses. Flowering time is an important trait with respect to drought adaptation, and it can shorten the life cycle and lead to drought escape or avoidance (Araus, Slafer, Reynolds, & Royo, 2002). In drought escape, plants flower early, a strategy that allows the parent plant to produce seeds before it is killed by drought (Fang & Xiong, 2014; Verslues & Juenger, 2011). However drought escape is fatal for leafy vegetables such as Chinese cabbage. Therefore, breeding Chinese cabbage varieties with high water use efficiency, which is simultaneously integrated with bolting-resistant genetic components, is the best solution to this issue.

In Arabidopsis, the flower-promoting gene *GIGANTEA* (*GI*), the florigen gene *FLOWERING LOCUS T* (*FT*), and*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) play a central role in the drought escape response (Blanvillain, Wei, Wei, Kim, & Ow, 2011; Riboni, Galbiati, Tonelli, & Conti, 2013). The phytohormone ABA is required for drought escape under long day (LD) conditions by activating the transcription of florigen genes (Riboni et al., 2013; Riboni, Robustelli Test, Galbiati, Tonelli, & Conti, 2016). For example, the ABA-responsive element (ABRE)-binding factors ABF3 and ABF4 induce *SOC1* transcription and promote flowering under drought conditions. Moreover, it has been shown that the activation of the florigen genes *HEADING DATE 3a* and *RICE FLOWERING LOCUS T1* , and the floral integrator OsMADS50 (an ortholog of SOC1), modulate the drought escape response through ABA-dependent and -independent pathways in rice (*Oryza sativa*) (Du et al., 2018). These studies suggest that ABA is a positive regulator of flowering in the drought escape response.

It has been shown that epigenetic mechanisms such as histone methylation and acetylation play an important role in the plant response to environmental conditions (Liu, Lu, Cui, & Cao, 2010; Luo et al., 2015). Down-regulation of protein N-terminal acetylation enhances Arabidopsis drought tolerance through the ABA pathway (Linster et al., 2015). The equilibrium between histone acetyltransferases (HATs) and histone deacetylases (HDACs) controls histone acetylation levels in nucleosomes, and this modification can also affect chromatin structure and gene expression in response to changes in the external environment (Shahbazian & Grunstein, 2007; Yuan, Liu, Luo, Yang, & Wu, 2013). Knocking out *GCN5* , which encodes a member of the HAT family, caused up-regulation of abiotic stress-inducible genes (Servet et al., 2008); the histone acetyltransferases HAM1 and HAM2 increase HIS4 acetylation levels in *FLC* chromatin to promote *FLC*expression and delay flowering time (Xiao et al., 2013). In Arabidopsis, the elongator HAT complex is involved in ABA, drought, and oxidative stress responses (Chen et al., 2006; Zhou, Hua, Chen, Zhou, & Gong, 2009; Versées, Groeve, & Lijsebettens, 2010). Histone deacetylase HDA5 promotes flowering time by repressing the expression of *FLC* and*MAF1* (Luo et al., 2015). HDA9 delays flowering time by repressing*AGL19* and thus promotes the expression of *FT* , especially under SDs (Kang, Jin, Noh, & Noh, 2015; Kim et al., 2016). Knocking out*HDA6* expression delayed flowering in both LD and SD conditions by derepressing *FLC* expression and reducing tolerance to ABA and drought stress (Kim et al., 2017; Wu, Zhang, Zhou, Yu, & Chaikam, 2008; Yu et al., 2011). Collectively, histone acetylation is involved in flowering and abiotic stress signaling; however, how these regulatory responses are coordinated via the various pathways, and the underlying mechanisms, are largely unknown.

Controlling the timing of flowering is important during the response stresses, but how the drought signal is integrated into the flowering pathways is poorly understood at present. In this study, we report that the expression of a *Brassica rapa* histone H4 gene (BrHIS4.A04,*Bra036357*) promotes flowering and decreases sensitivity to drought under normal growth conditions. Under drought stress conditions, the water deficit signal is probably translated to equilibrate the acetylation level of BrHIS4 at certain ABA and photoperiodic flowering gene loci, and therefore attenuates the expression to these genes to prevent premature bolting in

Chinese cabbage.

Materials and Methods

Plant materials and growth conditions

A collection of 194 Chinese cabbage inbred lines used was used for analysis of sequence variation and haplotyping of *BrHIS4.A04*.Detailed information on the 194 inbred lines is given in Su et al. (2018.)

The Arabidopsis ecotype Col-0 (Columbia) was used as the wild-type in this study. Arabidopsis seeds, including Col-0 and the *BrHIS4.A04* overexpression lines, were surface sterilized and incubated in the dark at 4°C for 4 days. The seeds were then sown on 0.5X MS salts medium solidified with 0.8% agar and grown at 22°C for 7 days. Seedlings were transferred to soil and cultivated in a growth chamber on a 16 h light/8 h dark cycle at 22°C. For drought treatment, 3-week-old seedlings of Arabidopsis or Chinese cabbage were deprived of water for 3 weeks. After the drought treatment, the plants were re-watered.

Plasmid construction and plant transformation

Total RNA was isolated from Chinese cabbage leaves using the RNAprep Pure Plant Kit (DP441, Tiangen). First-strand cDNA was synthesized using the Prime Script^{TM} reagent kit with gDNA Eraser (RR047A, Takara). The *BrHIS4.A04* and *BrVIN3.1* (Bra020445) coding sequences were amplified by PCR using first-strand cDNA as template. The coding sequences were then inserted into the *p* GADT7 and pGBKT7 plasmids, respectively. The *BrHIS4.A04* coding sequence was also inserted into the *p* MDC32 plasmid under control of the CaMV35S promoter. Primers used for cDNA amplification are given in Supplemental Table S1.

The *p* MDC32-BrHIS4.A04 recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 and used for plant transformation. The T_1-generation transgenic plants were selected on medium containing 15 mg/L hygromycin, and *BrHIS4.A04* expression was confirmed by semi-quantitative PCR. The primers used are given in Supplemental Table S1.

Yeast two hybrid (Y2H) assay

Young, healthy leaves from Chinese cabbage cultivar 'BY' (Yu, et al., 2016) were collected and used for total RNA extraction. The Chinese cabbage cDNA library in yeast was constructed using the MatePlate Library Construction System (630490, Clontech). The *BrVIN3.1* coding sequence was inserted into *p* GBKT7 and transformed into *Saccharomyces cerevisiae* strain Y2H. Transformants were selected on SD medium with –Trp dropout supplement. Yeast-mating was utilized to screen for BrVIN3-interacting proteins present in the Chinese cabbage cDNA library.

p GADT7-BrHIS4.A04, *p* GBKT7-BrVIN3.1, and the negative control plasmid were co-transformed into *Saccharomyces cerevisiae* AH109 cells using the lithium acetate method according to the manufacturer's protocol (630489, Clontech). The co-transformants were screened on double-dropout (-Trp/-Leu) medium at 30°C for 2 days. To detect protein-protein interactions, positive co-transformants were transferred to quadruple-dropout (-Trp/-Leu/-His/-Ade) medium and incubated at 30°C for 4 days. Potential interactions were further confirmed by X-gal staining.

Gene expression analysis

For the analysis of *BrHIS4.A04* expression during plant development, young leaves of the Chinese cabbage inbred line 'BY' were sampled at 10, 30, 50, 70, 90, and 120 days after planting in a growth chamber (16 h light/8 h dark cycle at 22°C). For the analysis of *BrHIS4.A04* expression in different tissues, seedlings, leaves, and roots were harvested from the 10-day plants, while inflorescences, flowers, and stems were sampled from the early flowering plants (~130 days after planting). For analysis of the flowering genes *FLC, GI, FT* , and *SOC1* , rosette leaves of 5-week-old Arabidopsis with or without drought treatment were collected before

flowering. For the analysis of drought- and ABA-responsive genes, we collected rosette leaves of 6-week-old Arabidopsis plants with or without drought treatment. Total RNA was isolated and gene expression was quantified by Real-Time PCR (RT-PCR) using SYBR Green PCR master mix (04887352001, Roche) on a LightCycler 480 RT-PCR system (Roche). *Actin2* was used as the internal control for normalization of gene expression. The primers used for RT-PCR are given in Supplemental Table S1.

Western blot

Rosette leaves were collected from 5-week-old Arabidopsis plants and ground to a powder in liquid nitrogen. The ground leaves were added to extraction buffer (50 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM phenylmethanesulfonyl fluoride and 1X protease inhibitor cocktail) and centrifuged at 14,000 g for 30 min at 4°C. Soluble proteins in the supernatant were fractionated by SDS-PAGE. Proteins were transferred to NC membrane (A19465264, GE healthcare) using the Semi-Dry Electrophoretic Transfer system (Bio-Rad). Anti-Histone H4 (ab10158, Abcam) and Anti-Histone H4 (acetyl K5+K8+K12+K16) (EPR16606, Abcam) antibodies were used as the primary antibodies. IRDye 680RD Goat anti-rabbit antibodies (926-68071, LI-COR) were used as the secondary antibody.

Chromatin Immunoprecipitation

ChIP experiments were performed using the Imprint Chromatin Immunoprecipitation Kit (CHP1-96RXN, Sigma) according to the manufacturer's protocol. Rosette leaves were collected from 5-week-old Arabidopsis plants and used for the ChIP experiments. Anti-Histone H4 (acetyl K5+K8+K12+K16) antibodies (EPR16606, Abcam) were used in the immunoprecipitations and normal mouse IgG (M8695, Sigma Aldrich) was used as the negative control. Quantitative measurements of gene fragments were performed using SYBR Green PCR master mix (04887352001, Roche) and the LightCycler 480 RT-PCR system (Roche). The *Actin2*gene was used as the internal gene expression control. The primers used are given in Supplemental Table S1.

Water content and ion leakage rate measurement

Rosette leaves from 6-week-old Arabidopsis seedlings were collected and weighed (fresh weight; FW). The leaves were then incubated at 50°C for 7 days and weighed again (dry weight; DW). Three replicates were measured for each transgenic line. Water content was calculated as follows:

Water content (%) = (FW-DW)/FW

The seventh rosette leaves from 6-week-old Arabidopsis seedlings were collected and incubated in 10 mL deionized water for 2 h. Conductance was measured using a conductivity meter and denoted as S1. The leaf in the 10 mL deionized water was then incubated at 100°C for 30 min and cooled to room temperature. Conductance was again measured and denoted as S0. Deionized water was used as the negative control and the conductance measurements were denoted as SC and SC0. The ion leakage rate was calculated as follows:

Ion leakage rate = (S1-SC)/(S0-SC0)

Measurements of stomatal density, stomatal aperture, and guard cell length

The middle section of a new, fully expanded rosette leaf was used to count the stomata and for aperture and guard cell length measurements. The abaxial epidermis was detached and photographed using a Nikon DS-RI microscope. Stomatal aperture and guard cell length were measured using Image J software, with at least 30 stomata measured from each transgenic line. The guard cell number and pavement cell number on each photograph were counted from at least 30 photographs of each transgenic line. Stomatal density was calculated as follows:

Stomatal density=stomatal guard cell number/pavement cell number.

Determination of flowering time, silique length, and seed number

Flowering time was determined by the number of rosette leaves present when the first bud appeared. At least 15 individual plants of each transgenic line were measured. Fully extended siliques from 7-week-old Arabidopsis plants were photographed, and the lengths were measured using Image J software. The number of seeds in each silique was then counted. At least 20 siliques from each transgenic line were measured.

Results

BrHIS4.A04 interacts with BrVN3.1

VIN3 binds to chromatin at the *FLC* locus (Sung & Amasino, 2004) and interacts with members of the conserved polycomb-group repressive complex 2 (PRC2) to perform its function (De Lucia, Crevillen, Jones, Greb, & Dean, 2008; Wood et al., 2006). In Chinese cabbage, it has been shown that one of the two *VIN3* genes, *BrVIN3.1* , was selected for in the breeding history of a late-bolting ecotype, spring Chinese cabbage (Su et al., 2018). To isolate proteins that interact with BrVIN3.1, we constructed a *B. rapa* leaf cDNA library in yeast. Using BrVIN3.1 as the 'bait' in a large-scale Y2H screening experiment, we isolated 11 potential interacting partners (**Table 1 and Figure 1 A, B**). Among these interactors, histone H4 has been previously reported to function in flowering time regulation (He, Michaels, & Amasino, 2003; Liu et al., 2010). Thus, we further verified the interaction between BrHIS4.A04 and BrVIN3.1 in the one-to-one yeast two-hybrid system (**Figure 1 A, B**).

Overexpressing *BrHIS4.A04* promotes flowering in Arabidopsis

HIS4 proteins are highly conserved in eukaryotes and show almost identical amino acid sequences within and between species (Alvarez & Loyola, 2017; Jiang & Berger, 2017; Sullivan & Landsman, 2003). In the*Brassica rapa* genome, there are 14 BrHIS4 homologs, of which BrHIS4.A04 shares 87% amino acid sequence similarity with Arabidopsis HIS4 (**Supplementary Figure 1A, C**). To investigate the expression patterns of these *BrHIS4* genes, RNA-seq data was generated from leaves harvested before flowering and during flowering (**Supplementary Figure 1B**). Eight of the 14 *BrHIS4* genes were detected (underlined in red in **Supplementary Figure 1B**), and we found that four of the eight (underlined in red and orange in**Supplementary Figure 1B**) showed differential expression in the two samples, including *BrHIS4.A04* (Bra035673). RT-PCR analysis was then performed on RNA extracted from multiple plant tissues at different developmental stages to explore the expression pattern of BrHIS4.A04 in the summer Chinese cabbage line 'BY' that can bolt within ~120 days without vernalization. Transcripts of*BrHIS4.A04* can be detected in all tissues or organs tested (including root, stem, inflorescence, flower, and 10-day-old seedlings) (**Figure 1D**) and, moreover, we found that the expression of*BrHIS4.A04* changed dynamically during plant growth, with the transcript level gradually rising prior to flowering and then decreasing after flowering (**Figure 1C**). To further study the function of*BrHIS4.A04* , the *BrHIS4.A04* coding sequence was inserted into the *p* MDC32 plasmid under control of the CaMV35S promoter and transformed into Arabidopsis Col-0. Two individual transgenic lines (hereinafter designated BrHIS4.A04$^{\mathrm{OE}}$) were used in a study in which the relative expression of *BrHIS4.A04* was significantly increased (**Supplementary Figure 2**). Homozygous*BrHIS4.A04*^{OE} plants showed no obvious morphological differences compared to wild-type plants at various stages of development (**Figure 1E and Supplementary Figure 3**). Both transgenic lines displayed normal rosette leaf development, and seed set was also found to be normal (**Figure 1E and Supplementary Figure 3**). However, in three independent experiments, we observed that*BrHIS4.A04*^{OE} plants display early flowering under normal LD conditions (**Figure 1E, F**). More specifically,*BrHIS4.A04*^{OE} plants flowered earlier (at ~6 rosette leaves) than did the control, and this difference was statistically significant (**Figure 1F**).

FLC acts as a floral repressor and inhibits flowering by directly repressing the promoters of the key flowering genes *FT* and*SOC1* (Lee et al., 2000). qRT-PCR analysis showed a significant decrease in expression of *FLC* , whereas the expression of the flowering activator *FT* increased in*BrHIS4.A04*^{OE} plants (**Figure 1G, H**

). These results suggest that BrHIS4.A04 negatively regulates *FLC* expression, and that it is also a positive regulator of *FT* .

Overexpression of *BrHIS4.A04* confers drought hypersensitivity

Flowering is initiated in response to the integration of both environmental cues and endogenous pathways. We noticed that in Arabidopsis, *AtHIS4* can be slightly induced by a 24-hour drought treatment in roots, but not by any other abiotic stresses, such as osmotic, salt, and heat stress (https://www.arabidopsis.org/servlets/TairObject?id=33482&type=locus). We thus exposed plants of the Chinese cabbage line BY to different abiotic stresses to investigate the expression profile of *BrHIS4.A04* under osmotic, salt, heat, and drought stress conditions. We found that *BrHIS4.A04* expression is induced by drought, suppressed by heat, and is unaffected under osmotic and salt stress conditions (**Supplementary Figure 4**). Moreover, the phenotypes of $BrHIS4.A04^{OE}$ and Col-0 plants in response to these stresses were recorded, and we found that the $BrHIS4.A04^{OE}$ plants are hypersensitive to drought in comparison with Col-0 (**Figure 2A**). There were no differences in the water content and ion leakage rate between the $BrHIS4.A04^{OE}$ and Col-0 plants under normal growth conditions; however, after drought stress, the water content of $BrHIS4.A04^{OE}$ plants was reduced to ~70% while the ion leakage rate increased to ~200% of that of the control plants (**Figure 2B, C**).

We then examined expression of the drought response marker gene *RD29b* (Yang et al., 2011). Under normal conditions, no difference in expression was found between the Col-0 and $BrH4IS.A04^{OE}$ plants. After drought treatment, *RD29b* expression was induced in both plants; however, the relative induction level was much lower in $BrHIS4.A04^{OE}$ plants (**Figure 2D**). It was previously reported that drought stress is accompanied by reactive oxygen species (ROS)generation (Gechev, Dinakar, Benina, Toneva, & Bartels, 2012). Expression of the *SOD* , *POD* , and *CAT12* genes that encode ROS scavenging enzymes was also detected under the different conditions. As we observed at *RD29b* , the relative degree of induction for all of the above three genes was less in the $BrHIS4.A04^{OE}$ plants compared with the control plants (**Figure 2E-I**). Taken together, these results suggest that the expression of *BrHIS4.A04* can attenuate the drought response in plants exposed to water deficit conditions.

BrHIS4.A04 leads to drought hypersensitivity independently of ABA

The plant response to drought is highly correlated with stomata because leaf water loss through transpiration is controlled by stomatal development (including the size and density of stomata on the epidermis) and behavior (the stomatal aperture) (Buckley, Sack, & Farquhar, 2017). We therefore first investigated stomatal size and density to understand the reason for the higher water loss observed in $BrHIS4.A04^{OE}$ plants; however, no difference was found between the $BrHIS4.A04^{OE}$ and Col-0 plants with or without drought treatment (**Figure 3A-H**). Stomatal movement was then measured, and the results showed that drought induced dramatic stomatal closure in wild-type plants, but the stomatal apertures were only slightly decreased in $BrHIS4.A04^{OE}$ plants (**Figure 3I**).

ABA is an important mediator between drought stress and stomatal movement (Leung & Giraudat, 1998; Lim, Baek, Jung, Kim, & Lee, 2015). To determine whether BrHIS4.A04 induces drought hypersensitivity through the ABA pathway, genes involved in ABA biosynthesis (*ABA1* and *NCED3*) and signaling (*ABI1* and *MYB2*) were analyzed. As in the drought response genes described above, the expression of the four ABA genes was higher in $BrHIS4.A04^{OE}$ plants compared with Col-0 under normal conditions, and expression was induced in both plants after drought treatment. However, the induction was to a much lesser degree in $BrHIS4.A04^{OE}$ plants (**Figure 3J-M**). These results suggest that overexpressing *BrHIS4.A04* in Chinese cabbage leads to drought hypersensitivity dependently of ABA.

BrHIS4.A04 prevents premature flowering under drought conditions

Since BrHIS4.A04 is involved in both the drought response and flowering control, we asked whether BrHIS4.A04 plays a role in integrating the drought response with flowering regulation. The flowering times

of the*BrHIS4.A04*^{OE} and Col-0 plants grown under drought stress were then recorded (**Figure 4A, B**). We found that the drought-treated Col-0 plants bolted earlier than plants grown under normal conditions as expected; however, the drought-treated*BrHIS4.A04*^{OE} plants bolted at roughly the same time, if not slightly later than, the plants grown under normal condition (**Figure 4B**). This result showed that drought had a much stronger effect on wild-type plants, but no effect or only a slight effect on the *BrHIS4.A04*^{OE} plants.

We next examined the expression of *FLC* , *GI* , *FT* , and*SOC1* in plants grown under both normal and drought conditions. Under normal conditions, the over-expression of *BrHIS4.A04* resulted in increased levels of *GI* , *FT* and *SOC1* transcription (**Figure 4D-E**), and slightly reduced levels of*FLC* -specific mRNA (**Figure 4F**), suggesting that the early flowering of *BrHIS4.A04*^{OE} plants grown under normal conditions mainly depends on the photoperiodic flowering pathway, which was also reported by Riboni et al. (2016). After drought treatment, a slight reduction in *FLC* expression and increases in the expression of *GI* , *FT* and *SOC1* were found in Col-0 plants (**Figure 4C-F**) as reported by Riboni et al. (2013)**.** Intriguingly, however, we found that there was no increase in the expression of the *GI* , *FT* , and *SOC1* genes in *BrHIS4.A04*^{OE} plants (**Figure 4D-F**), which is consistent with the observation that there was no difference in flowering time between the normally-grown and drought-treated *BrHIS4.A04*^{OE} plants. These results suggest that BrHIS4.A04 can prevent premature flowering in Chinese cabbage under drought conditions, mainly by maintaining the expression of photoperiodic flowering genes, which is further supported by the fact that although expression of *FLC* was dramatically induced after drought in *BrHIS4.A04*^{OE} plants, the flowering time did not change (**Figure 4B, C**).

Overexpressing *BrHIS4.A04* leads to a higher chromatin acetylation level in plants

It has been widely reported that histone H4 acetylation is involved in regulating flowering time and the drought response (He et al., 2003). Considering the possibility that BrHIS4.A04 may be one of the hubs of the two physiological activities, we wondered whether the two processes are coordinated via the acetylation of histone H4. Total protein was then extracted from the leaves of plants grown with or without drought treatment, and a crude profile of H4 acetylation level was determined by Western blotting using an anti-H4 (acetyl K5+K8+K12+K16) antibody. Under normal growth conditions, a higher level of H4 acetylation was found in*BrHIS4.A04*^{OE} plants in keeping with its higher histone H4 protein level detected using the anti-H4 antibody. After drought treatment, the H4 acetylation level increased dramatically in Col-0 plants as expected; however, no increase was found in*BrHIS4.A04*^{OE} plants (**Figure 5A**).

We then examined H4 acetylation at four genetic loci; the ABA genes*ABI1* and *MYC2* and the flowering genes *FLC* and*FT* . Chromatin immunoprecipitation assays with anti-H4 (acetyl K5+K8+K12+K16) antibody were performed followed by RT-PCR (**Figure 5B-E and Supplementary Figure 5**). Under normal growth conditions, the levels of H4 acetylation at all the tested loci were much higher in *BrHIS4.A04*^{OE} plants when compared with Col-0 plants (**Figure 5B-E**). This result is consistent with their expression profiles and the consequent early-flowering and drought-sensitive phenotypes of the*BrHIS4.A04*^{OE} plants. After drought treatment, the H4 acetylation levels of these genes increased dramatically in Col-0 plants, but remained at about the same level in*BrHIS4.A04*^{OE} plants grown under both normal and drought conditions (**Figure 5B-E**). This observation is in keeping with the hypothesis that BrHIS4.A04 can prevent premature flowering by attenuating the expression of drought and photoperiodic flowering genes.

Discussion

BrHIS4.A04 is a non-canonical histone H4

In the nuclei of eukaryotic cells, chromatin is the physiological template for various genetic processes. The basic unit of chromatin is the nucleosome, a structure composed of an octamer of the core histone proteins H2A, H2B, H3, and H4, around which is wrapped 146 base pairs of chromosomal DNA. These proteins are highly conserved across eukaryotic species (Alvarez & Loyola, 2017; Sullivan & Landsman, 2003; Weber & Henikoff, 2014). All four types of histones are encoded by multiple genes; for example, there are eight *AtHIS4*

genes with different nucleotide sequences, but all eight genes encode single proteins with identical amino acid sequences, suggesting its conserved role in evolution (Tenea et al., 2009). Protein sequence alignment revealed that the protein encoded by *BrHIS4.A04* shares 87% amino acid sequence homology with that encoded by *AtHIS4* (**Supplementary Figure 1C**). Furthermore, we found that the expression of*BrHIS4.A04* is induced during growth and by drought in Chinese cabbage (**Figure 1C, Supplementary Figure 3**). Thus, the conserved features of *BrHIS4.A04* and its induced expression in response to drought, together with the functional studies shown in the Results section hint at its non-canonical role compared to other BrHIS4 proteins. Histones function to control the structure and accessibility of the chromatin environment by altering the biochemical properties of the nucleosome or through the recruitment of distinct binding partners (Jiang & Berger, 2017; Melters et al., 2019; Weber & Henikoff, 2014). One such method of histone-mediated control comes from the exchange of canonical histones with non-allelic histone variants, which alter the fundamental structure and stability of the nucleosome. H2A.Z is one of the most enigmatic of these histone variants (Kawashima et al., 2015). It has been reported that H2A.Z within gene bodies is correlated with genes that respond to environmental stresses (Dong et al., 2018; Sura et al., 2017). One potential scenario is that BrHIS4.A04 gained some similar features of H2A.Z in the evolution of Chinese cabbage, and the amino acid variant of BrHIS4.A04 might facilitate it to specially target some environmentally responsive genes. However this hypothesis needs to be further tested by comparing the genome-wide target genes of BrHIS4.A04 and other BrHIS4 homologues which can be identified by the ChIP-seq method.

H4 acetylation is one of the hubs that connects the drought response and flowering regulation

Resistance to drought involves global reprogramming of transcription, hormone signaling, and chromatin modification in plants (Fang & Xiong, 2014). However, the ways in which these regulatory responses are coordinated via the various pathways are largely unknown. Hwang et al. (2019) reported that the ABA-responsive element (ABRE)-binding factors ABF3 and ABF4 are rapidly induced when the plant is exposed to drought stress, and that ABF3 and ABF4 interact with NF-YCs to promote flowering by inducing SOC1 transcription. Here, together with our findings, we report an essential drought-responsive network in which plants trigger the ABA signaling to stimulate flowering to confer drought response, and the change in H4 acetylation at certain ABA and flowering gene loci is the hub of the network. In Arabidopsis, we observed that mutation of histone H4 deacetylase HDA6 resulted in enhanced drought tolerance and late flowering (Wu et al., 2008). Further study showed that HDA6 directly represses the acetate biosynthesis pathway under normal conditions (Kim et al., 2017). In addition, exogenous acetic acid can be converted to acetyl-CoA and used as a substrate for histone acetylation to increase the acetyl-H4 level and boost drought tolerance in plants. Therefore, we proposed that the drought-induced activation of acetate biosynthesis potentially leads to genome-wide H4 acetylation, which connects fundamental metabolism, epigenetic regulation, and hormone signaling, and ultimately affects plant environmental adaptation. Besides, compared with chromatin modifiers, there are quite a few studies that discuss the role of their histone targets, except for H2A.Z, in which they are used as epigenetic indicators in most cases. H4 acetylation is often associated with chromatin remodeling during gene activation, but as far as we know, there are no studies that describe the effect of H4 acetylation on *FT* expression. The results of our study suggest that H4 acetylation positively regulates the expression of*FT* , and a proper acetylation of BrHIS4 at the *FT* locus can keep it functioning within a reasonable range.

BrHIS4.A04 is selected in Chinese cabbage breeding?

Mining elite alleles for drought resistance and late flowering is important for the improvement of cultivated Chinese cabbage and selection for market demand. Sequence analysis of *BrHIS4.A04*showed 28 SNPs in the gene body, with 24 SNPs in the promoter and the other four in the coding region that did not cause changes in the amino acid sequence. To gain further insight into the allelic differentiation of *BrHIS4.A04* , we extended our study in the 194 Chinese cabbage accessions reported by Su et al. (2018, 2019). Two haplotypes of*BrHIS4.A04* were identified in the 194 lines; Hap1 was the major haplotype present in 182 accessions, while only 12 accessions carry Hap2 (**Supplementary Figure 6** A, B). Phylogenetic analysis

showed that the 194 line can be classified into four different groups (Su et al., 2018)

The Hap1-carrying lines were found to be distributed among all four groups, while the Hap2 lines were only present in the relatively primitive Aut1 and summer groups (**Supplementary Figure 6C**). Taking geography into account, we noticed that seven of the 12 Hap2 lines originate from Tianjin, China (**Supplementary Figure 6C, red circles**). These results are consistent with the fact that Chinese cabbage germplasm from Tianjin has been reported to be salt and drought tolerant due to its long breeding history on mildly saline and alkaline soils. The drought tolerance of the seven lines was further studied, and they indeed showed higher survival rates after rehydration (**Supplementary Figure 6** D). However, the significance of this result can be statistically evaluated only after more varieties from Tianjin are collected and introduced into the study.

Taken together, we report here that BrHIS4.A04 can prevent premature flowering mainly through its action on the photoperiodic flowering pathway by attenuating ABA signaling under drought conditions. Since$BrHIS4.A04^{OE}$ plants displayed no phenotypes related to vegetative and reproductive development in response to drought, we think that our findings will contribute to the fine-tuning of flowering time in crops with no growth penalty by genetic engineering or other breeding methods.

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Table1 Potential BrVIN3.1 interaction partners isolated from a yeast-two hybrid screen of a cDNA library prepared from Chinese cabbage mRNA

Gene	Function
Bra033285	60S acidic ribosomal protein p1-3
Bra006691	zinc finger protein ZAT12
Bra016459	catalase-3
Bra002054	AP-1 complex subunit sigma-1
Bra006824	VIN3-like (VERNALIZATION INSENSITIVE 3)
Bra035673	histone H4
Bra001028	40S ribosomal protein S19-1
Bra013206	dehydrin family protein HiRD11
Bra028408	RAD23C
Bra021078	VRN2 (REDUCED VERNALIZATION RESPONSE 2)
Bra018120	microtubule-associated protein RP/EB family member 1A

Figure legends

Figure 1 The BrVIN3.1-interacting protein BrHIS4.A04 promotes flowering in Arabidopsis.

(A) One-to-one yeast two hybrid assay of the interaction between AD-BrHIS4.A04 and BD-BrVIN3.1. (B) The interaction between AD-BrVIN3.1 and BD-BrHIS4.A04 was confirmed by X-gal staining. (C, D) Expression analysis of *BrHIS4.A04* during different growth stages (C) and in different tissues of Chinese cabbage (D) Transcripts of*BrHIS4.A04* can be detected in all tissues or organs tested, including roots (r), stems (st), leaves (l), inflorescences (in), flowers (f) and 10-day-old seedlings (s), and it increased gradually be-

fore flowering and decreased after flowering. (E) Six-week-old*BrHIS4.A04*^{OE} plants flower earlier than the Col-0 controls under long day (16 h/8 h day/night, LD) conditions. (F) Numbers of rosette leaves on Col-0 and*BrHIS4.A04*^{OE} plants at bolting under LD conditions; *BrHIS4.A04*^{OE} plants bolted earlier than Col-0. (G to H) Real-time (RT)-PCR analysis of *FLC* and*FT* expression in 5-week-old Col-0 and*BrHIS4.A04*^{OE} plants before flowering;*FLC* expression was reduced and *FT* expression was increased in BrHIS4.A04$^{\mathrm{OE}}$ plants. Error bars indicate ± standard error (SE) of the mean. Columns labeled with different letters are statistically different according to the Student's t-test (P <0.05).

Figure 2 Overexpression of *BrHIS4.A04* confers drought hypersensitivity

(A) *BrHIS4.A04*^{OE} plants are more severely affected than Col-0 plants after 3 weeks of drought treatment. (B) Water content and (C) ion leakage rate in 6-week-old Col-0 and*BrHIS4.A04*^{OE} plants with or without drought treatment. There were no differences in the water contents and ion leakage rates between the *BrHIS4.A04*^{OE} and Col-0 plants grown under normal conditions; however, after drought stress, the water contents of the *BrHIS4.A04*^{OE}plants were reduced to ~70% of the control (Col-0), while the ion leakage rates increased between 5- and 6-fold compared to the control plants. (D-G) RT-PCR analysis of drought and ROS responsive genes. The relative degree of induction in the drought (D) and ROS (E to G) response genes was less in *BrHIS4.A04*^{OE}plants compared to Col-0. Error bars indicate ± standard error (SE) of the mean. Columns labeled with different letters are statistically different according to the Student's t-test (P <0.05).

Figure 3 BrHIS4.A04 leads to drought hypersensitivity dependently of ABA

Images of stomata from 6-week-old Col-0 and*BrHIS4.A04*^{OE} plants grown under normal conditions (A to C) or after 3 weeks of drought treatment (D to F). Scale bars represent 50 μm. (G) Stomatal density, (H) guard cell length, and (I) stomatal aperture size were measured in 6-week-old Col-0 and*BrHIS4.A04*^{OE} plants with or without drought treatment. *BrHIS4.A04*^{OE} plants had larger stomatal apertures under drought conditions. RT-PCR analysis of ABA synthesis (J, K) and ABA signaling (L, M) genes showed that the expression of the four ABA genes was higher in*BrHIS4.A04*^{OE} plants compared with Col-0 plants grown under normal conditions, and was induced in both plants after drought treatment. However, the induction was to a much lesser degree in*BrHIS4.A04*^{OE} plants. Error bars indicate ± standard error (SE) of the mean. Columns labeled with different letters are statistically different according to the Student's t-test (P <0.05).

Figure 4 BrHIS4.A04 prevents premature flowering under drought conditions.

(A) Flowering in 6-week-old Col-0 and*BrHIS4.A04*^{OE} plants grown under normal and drought conditions. For drought treatment, 3-week-old Arabidopsis seedlings were deprived of water for three weeks. (B) Rosette leaf numbers of Col-0 and *BrHIS4.A04*^{OE} plants at bolting. The drought-treated*BrHIS4.A04*$^{O\ \mathrm{E}}$ plants bolted at roughly the same time as the normal-grown plants. (C to F) RT-PCR analysis of flowering genes in 5-week-old Col-0 and*BrHIS4.A04*^{OE} plants. Under normal growth conditions, the expression of *BrHIS4.A04*^{OE}resulted in increased levels of *GI* -, *FT* , and*SOC1* -specific mRNA (**D, E**), and slightly reduced the mRNA level of *FLC* ; after drought treatment, a slight reduction in*FLC* and increases in *GI, FT* , and *SOC1* expression were found in Col-0 plants (**C-F**). However, there was no increase in the expression of *GI, FT* , and *SOC1* in*BrHIS4.A04*^{OE} plants. Error bars indicate ± standard error (SE) of the mean. Columns labeled with different letters are statistically different according to the Student's t-test (P <0.05).

Figure 5 Overexpressing *BrHIS4.A04* leads to increased chromatin H4 acetylation in Arabidopsis plants.

(A) H4 acetylation levels in five-week-old Col-0 and*BrHIS4.A04*^{OE} plants with or without drought were determined by Western blotting using anti-H4 (acetyl K5+K8+K12+K16) antibody. The H4 protein level was used as an internal control. Under normal growth conditions, a higher level of H4 acetylation was observed in *BrHIS4.A04*^{OE} plants. After drought treatment, the BrHIS4.A04 acetylation level increased dramatically in Col-0 plants; however, no increase was found in*BrHIS4.A04*^{OE} plants. (B-F) The BrHIS4.A04 acetylation level at the *ABI1, MYC2, FT* , and *FLC* loci was determined by ChIP followed by RT-PCR analysis.

Under normal growth conditions, the levels of H4 acetylation at all the tested loci were much higher in *BrHIS4.A04*^{OE} plants compared to Col-0 plants. After drought treatment, the H4 acetylation levels of these genes increased dramatically in Col-0, but remained at about the same level as in the*BrHIS4.A04*$^{O\ E}$ plants grown under normal conditions. Error bars indicate ± standard error (SE) of the mean. Columns labeled with different letters are statistically different according to the Student's t-test (P <0.05).

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