

From the laboratory to the field: assaying histone methylation at *FLOWERING LOCUS C* in naturally growing *Arabidopsis halleri*

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Gene regulatory mechanisms are often defined in studies performed in the laboratory but are seldom validated for natural habitat conditions, i.e., *in natura*. Vernalization, the promotion of flowering by winter cold, is a prominent naturally occurring phenomenon, so far best characterized using artificial warm and cold treatments. The floral inhibitor *FLOWERING LOCUS C* (*FLC*) gene of *Arabidopsis thaliana* has been identified as the central regulator of vernalization. *FLC* shows an idiosyncratic pattern of histone modification at different stages of cold exposure, believed to regulate transcriptional responses of *FLC*. Chromatin modifications, including H3K4me3 and H3K27me3, are routinely quantified using chromatin immunoprecipitation (ChIP), standardized for laboratory samples. In this report, we modified a ChIP protocol to make it suitable for analysis of field samples. We first validated candidate normalization control genes at two stages of cold exposure in the laboratory and two seasons in the field, also taking into account nucleosome density. We further describe experimental conditions for performing sampling and sample preservation in the field and demonstrate that these conditions give robust results, comparable with those from laboratory samples. The ChIP protocol incorporating these modifications, “Field ChIP”, was used to initiate *in natura* chromatin analysis of *AhgFLC*, an *FLC* orthologue in *A. halleri*, of which a natural population is already under investigation. Here, we report results on levels of H3K4me3 and H3K27me3 at three representative regions of *AhgFLC* in controlled cold and field samples, before and during cold exposure. We directly compared the results in the field with those from laboratory samples. These data revealed largely similar trends in histone modification dynamics between laboratory and field samples at *AhgFLC*, but also identified some possible differences. The Field ChIP method described here will facilitate comprehensive chromatin analysis of *AhgFLC* in the future to contribute to our understanding of gene regulation in fluctuating natural environments.

Key words: *Arabidopsis halleri*, chromatin immunoprecipitation, *FLOWERING LOCUS C*, histone modification, natural plant population

INTRODUCTION

Natural environments generate complex, often noisy,

signals; yet living organisms, especially sessile organisms, including plants, are endowed with systems capable of decoding noisy signals to ensure their survival. Molecular approaches to understanding such response systems generally address the effect of a single type of signal at a time on gene expression, and have been mainly laboratory-based. However, studies in natural habitats, *in*

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natura, are crucial for validating gene regulatory mechanisms defined under laboratory conditions and are, furthermore, important for addressing novel questions that are specific to natural conditions (Kudoh and Nagano, 2013; Kudoh, 2015). For example, how does the gene regulatory system behave robustly under natural conditions, and how are useful environmental cues extracted from noisy signals?

One of the best-known, naturally occurring environmental responses in plants is vernalization, the promotion of flowering by prolonged cold of winter. A comprehensive *in natura* gene expression profile of the central gene of this process, *FLOWERING LOCUS C* (*FLC*), was reported for a two-year complete seasonal cycle of *Arabidopsis halleri* subsp. *gemma* (*A. halleri*, hereafter) (Aikawa et al., 2010). Those quantitative gene expression data demonstrated that *A. halleri FLC* (*AhgFLC*) expression follows the seasonal trend of the temperature. Modelling of the gene expression data versus environmental temperature led to the conclusion that the seasonal gene expression was controlled by temperatures in the preceding six weeks, which in turn led the authors to postulate the existence of a long-term memory mechanism that functions to filter out short-term noise (Aikawa et al., 2010). While the basis of this mechanism remained largely unknown, a wealth of molecular data available from laboratory studies of *Arabidopsis thaliana* shows that *A. thaliana FLC* is regulated by a universal cellular memory machinery based on two major protein complexes with antagonistic activities: Trithorax complex catalyses the trimethylation of lysine 4 of histone H3 tails (H3K4me3), which is involved in maintenance of active gene transcription, while Polycomb complex catalyses the trimethylation of lysine 27 of histone H3 tails (H3K27me3), which is involved in maintenance of repressed gene activity (Buzas et al., 2012; Song et al., 2012).

Both H3K4me3 and H3K27me3 have been reported to change dynamically at the *FLC* locus during different stages of vernalization in the annual *A. thaliana* (Sung and Amasino, 2004; Finnegan and Dennis, 2007; Yang et al., 2014). Before cold exposure, the *FLC* transcription start site (TSS) is marked with H3K4me3 and the gene is actively transcribed to repress flowering (Pien et al., 2008; Tamada et al., 2009). During cold exposure, H3K27me3 accumulates increasingly at the TSS in a manner dependent on the extent of the cold exposure, and this process is associated with the quantitative degree of silencing of *FLC* (Sung et al., 2006; Finnegan and Dennis, 2007; Angel et al., 2011). After plants are returned to warm conditions, H3K27me3 accumulates across the entire *FLC* gene body, and the silencing is maintained (Finnegan and Dennis, 2007; Angel et al., 2011) until the end of the life cycle. In plants with a perennial life cycle, *FLC* silencing occurs transiently during flowering, and

FLC is up-regulated again to repress flowering during successive vegetative growth (Wang et al., 2009; Aikawa et al., 2010). A laboratory study of the perennial *Arabidopsis alpina* showed that H3K27me3 is deposited at the *A. alpina FLC* locus when the gene is repressed, and is removed when the gene is activated (Wang et al., 2009). However, exactly how such dynamics of H3K4me3 and H3K27me3 control memory is still unknown. Nevertheless, because chromatin-driven gene expression control is generally considered to be the main candidate mechanism for long-term memory underlying seasonal expression of *AhgFLC*, it is important to obtain *in natura* profiles of H3K4me3 and H3K27me3 at *AhgFLC*.

The main aim of this study was to modify a standard chromatin immunoprecipitation (ChIP) protocol, normally used with laboratory samples, to make it suitable for analysis of histone modifications of field samples, and then to quantify the *in natura* patterns of histone modifications at *AhgFLC*. ChIP includes a number of steps, all performed in the laboratory (Orlando, 2000; Haring et al., 2007; Collas, 2010). To keep the histones in contact with the DNA throughout the ChIP procedure, the laboratory tissues are first infiltrated with a cross-linking agent (e.g., formaldehyde) for a short time, and then immediately frozen in liquid N₂. After chromatin is thus cross-linked, it is fragmented (e.g., mechanically by sonication) and incubated with antibodies against specific histone modifications. Chromatin fragments bound to the antibodies are collected using protein A/G beads. Finally, DNA is eluted from the beads and extracted after the cross-linking is reversed.

In the field, because plants are often widely scattered, sampling may take a long time. Moreover, transport of liquid N₂ to the field may be a serious traffic hazard. We therefore modified the initial steps of ChIP so as to perform them in the field, rather than in the laboratory. In this manuscript, we refer to this modified ChIP procedure as “Field ChIP”. The flow chart of Field ChIP is highlighted in Fig. 1A, and a hands-on protocol is provided in Supplementary Text S1. We monitored the behaviour of putative internal control genes for each of H3K4me3 and H3K27me3 to validate them as appropriate normalization controls. The validation was performed under two conditions each for the laboratory-controlled cold (non-vernalized and vernalized) and for the field (autumn and winter). To control for possible variations in the density of nucleosomes under these conditions, we performed histone H3 ChIP along with the ChIPs for histone lysine methylations. Using the Field ChIP method, here we report initial field data on changes of H3K4me3 and H3K27me3 at *AhgFLC* between autumn and winter in a natural population of *A. halleri* in Hyogo prefecture, Japan, and compare these results with those of vernalization experiments in the laboratory.

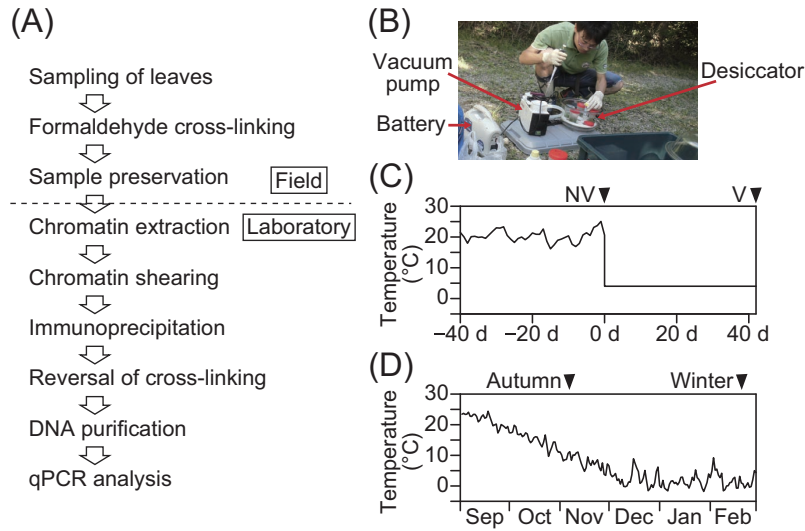


Fig. 1. Outline of Field ChIP and the temperature regimes of the vernalization experiments. (A) A flow chart of Field ChIP. The first three steps are performed in the field. (B) A photograph showing formaldehyde cross-linking of leaf samples in the field, indicating the portable instruments used in this study (red arrows). (C) Temperature regime of the vernalization experiment. The x-axis represents days before or after transfer to cold (0 d is the day of transfer: October 12, 2013). Temperatures from -40 to 0 d represent field temperatures before transplantation. Non-vernalized (NV) and vernalized (V) laboratory samples were obtained at the time points indicated by triangles above the graph. (D) Temperature regime in the field. Autumn (November 6, 2012) and winter (February 19, 2013) field samples were obtained at the time points indicated by triangles above the graph. In (C) and (D), changes in daily mean temperature are plotted.

MATERIALS AND METHODS

Plant materials and growth conditions Plant materials used in this study were collected from a natural population of *A. halleri* at Omoidegawa river, Naka-ku, Taka-cho, Hyogo prefecture (35°06' N, 134°55' E, altitude 190–230 m). For controlled cold experiments, six naturally growing *A. halleri* plants were transferred from the field to the laboratory on October 8, 2013, a time when *Ahglc* is highly expressed (Aikawa et al., 2010), transplanted into pots and grown on outdoor pot shelves at the Center for Ecological Research, Kyoto University (34°58' N, 135°57' E, altitude 152 m). Fully expanded young leaves were harvested from three of these plants after a 4-day period of growth adjustment in the outdoor pots (non-vernalized, NV) and the remaining three plants were kept at constant cold, 4 °C, for six weeks prior to the second sampling (vernalized, V) (Fig. 1C). For both NV and V samples, two sets of three leaves (0.1 g per leaf × 3 leaves × 2 sets) were harvested from each plant, and used for comparisons in other examinations. Field samples were collected in two seasons: in autumn (November 6, 2012), when *Ahglc* is actively expressed, and in winter (February 19, 2013), when *Ahglc* is repressed (Aikawa et al., 2010, Fig. 1D). For each sampling date, we randomly selected forty plants, and harvested one fully expanded young leaf from each plant (0.1 g per plant). We pooled ten leaves for each ChIP sample (1 g

per replicate) and obtained four biological replicates per collection date. We analysed leaf samples because they are the most accessible tissues of plants year-round in the field, and because leaf samples were analysed in a related previous study (Aikawa et al., 2010).

Formaldehyde cross-linking Harvested leaves were cut in half and soaked in 1% formaldehyde in PBS buffer. Vacuum infiltration was conducted twice, for 5 min each, at ambient temperature (laboratory or field). To quench the cross-linking reaction, glycine was added to a final concentration of 125 mM and vacuum infiltration was conducted for an additional 5 min. Cross-linked samples were washed with PBS, and then either kept in PBS on ice for 7 h before freezing (for sample preservation) or immediately frozen in liquid N₂, before storage at -80 °C until chromatin extraction.

Chromatin immunoprecipitation ChIP experiments were conducted following the protocol of Gendrel et al. (2005) with some modifications. Extracted chromatin was sonicated eight times for 15 s each using a Q700 Sonicator (Qsonica, Newtown, CT, USA) at 10% power output and samples were placed on ice for at least 1 min between each sonication; the total time of incubation on ice ranged from 20 to 30 min. Samples were centrifuged at 4 °C for 5 min at 12,000 g and the supernatant was diluted in ChIP dilution buffer (3.0 ml of buffer per 1 g sample).

This chromatin lysate was then incubated with Dynabeads Protein G (Novex, Waltham, MA, USA) at 4 °C for 1 h with rotation for pre-clearing. Pre-cleared chromatin was then incubated with antibody for 5 h. Antibody dilutions were as follows: 1:500 for anti-trimethyl-histone H3 (Lys4) (#07-473, Millipore, Billerica, MA, USA) and anti-trimethyl-histone H3 (Lys27) (#07-449, Millipore) and 1:1000 for anti-histone H3 (#ab1791, Abcam, Cambridge, UK). Immunoprecipitated complexes were collected with Dynabeads Protein G by incubation for 2 h, and the beads were then washed sequentially with low salt buffer, high salt buffer, LiCl buffer and TE buffer. Immunoprecipitated chromatin was eluted with elution buffer, and cross-linking was reversed by heating at 65 °C for 12 h and proteinase K treatment at 45 °C for 1 h. After phenol/chloroform extraction, DNA was ethanol-precipitated, and resuspended in 50 µl of TE buffer. The buffer compositions are described in Gendrel et al. (2005).

RT-qPCR and ChIP-qPCR qPCR was carried out with POWER SYBR Green PCR Master Mix (#4367659, Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions, using the 7300 Real-time PCR System (Applied Biosystems). We designed the primers for reference genes based on draft sequences of the corresponding genes (Supplementary Text S2, Supplementary Fig. S1) except for *AhgFLC* primers, which were designed based on the genomic sequence (GenBank accession number: KC505459.1). All primers used for qPCR are listed in Table 1. To perform absolute quantification, we used a standard curve with five concentrations of standard samples. We confirmed that all primers in this study efficiently amplified their targets within this range. qPCR of all samples was performed in duplicate, and the means were used for calculations.

For RT-qPCR, young leaves of *A. halleri* were sampled in autumn and in winter at our field site. For the evaluation of reference genes, autumn and winter samples were collected on October 4, 2012 and February 26, 2013, respectively (six replicates from separate individuals per sampling date); for the quantification of *AhgFLC* expression, they were collected on November 6, 2012 and February 19, 2013, respectively (four replicates). RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and quantified using Qubit Fluorometer and Qubit RNA HS Assay Kits (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 200 ng of RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Approximately 10 ng of RNA was used in each qPCR, and qPCR for each sample was performed in duplicate. Normalization was done using a standard cDNA sample produced from laboratory-grown NV plants.

For ChIP-qPCR, we analysed samples described in the "Plant materials and growth conditions" section. We

Table 1. List of primers used for RT-qPCR and ChIP-qPCR

Gene	Experiment	Primer sequence
<i>AhgPP2AA3</i>	RT-qPCR	5'-GTATGCACATGTTTTGCTTCCAC-3'
		5'-CAACCAAGTCATTTCTCCCTCATC-3'
	ChIP-qPCR	5'-CAGCGTAATCGGTAAATCTCG-3'
		5'-CCGTCCCAATCTAGAGAGAACTAC-3'
<i>AhgRBP45B</i>	RT-qPCR & ChIP-qPCR	5'-TCGCAGATGGTTTCTGCTAAAG-3'
		5'-CGGGATAGGAGCGTTGTTG-3'
<i>AhgUBP1A</i>	RT-qPCR	5'-CGATCTCAGTCCCTGCTCTGT-3'
		5'-CCTGCTTGCCCTTAGAGAATGTTG-3'
	ChIP-qPCR	5'-TTCGTGGGTTTTAATCACTGAGC-3'
		5'-ACGAAACACAAGCCCACAATC-3'
<i>AhgACT2</i>	RT-qPCR & ChIP-qPCR	5'-GCGACCAGACAGAGAAAGAAGG-3'
		5'-GATGGAGAAAAGCGGAAGAAGA-3'
<i>AhgSTM</i>	ChIP-qPCR	5'-ATCAGTCACACCACCAACAAAGTAG-3'
		5'-AGAGAATAAGCAGGGGCACAAG-3'
<i>AhgAG</i>	ChIP-qPCR	5'-CTTGAGGAAATCTGGGAGAGGA-3'
		5'-CTTCAGCATCACAAAGAACAGAGAG-3'
<i>AhgFUS3</i>	ChIP-qPCR	5'-ATTCTCAACGGAGCCCAAC-3'
		5'-AACCTCCAACGACACTCTCTC-3'
<i>AhgFBOX</i>	ChIP-qPCR	5'-AAGAGAATAGCCAGACCCAATCC-3'
		5'-TCCGATACGCAACGAAACC-3'
<i>AhgFLC</i>	RT-qPCR	5'-CGGCGGTTAAAATCAAATC-3'
		5'-CAAACGCTCGCCCTTATC-3'
<i>AhgFLC</i> 5' UTR	ChIP-qPCR	5'-AAAAGGAAAGAGAAGAACGCTTAG-3'
		5'-AGAGGGCTTTATTCCTTAGTTTG-3'
<i>AhgFLC</i> 1st intron	ChIP-qPCR	5'-GAAAACCGACAATCACACAACC-3'
		5'-TAGAGGCACCAAGAAACAAGG-3'
<i>AhgFLC</i> 5th intron	ChIP-qPCR	5'-TCTTAACGAGCTTGACACATC-3'
		5'-AAGACCGACACTTTCAGCAAATAC-3'
<i>AhgFLC</i> 7th exon	ChIP-qPCR	5'-CATCATGTGGGAGCAGAAGCT-3'
		5'-CGGAAGATTGTCGGAGATTTG-3'

used input DNA to generate a standard curve.

Data normalization Expression of candidate reference genes was compared without normalization (Fig. 2). We performed H3 ChIP (see Fig. 3 for candidate reference genes) along with the ChIPs for histone lysine methylations to confirm that differences in both H3K4me3 and H3K27me3 levels reflected actual modification differences and not histone density variation at the regions analysed under our conditions. The H3K4me3 and H3K27me3 ChIPs were normalized as follows: in Fig. 4, the absolute amount of H3K4me3/H3K27me3 ChIP DNA, expressed as % of input, was divided by the absolute amount of H3 ChIP DNA, expressed as % of input at the same region; in Figs. 5B, 5D and 6B, *AhgPP2AA3* was used as an internal control for calculating H3K4me3 levels at *AhgFLC*; in Figs. 5C, 5E and 6C, *AhgSTM* was used as an internal control for calculating H3K27me3 levels at

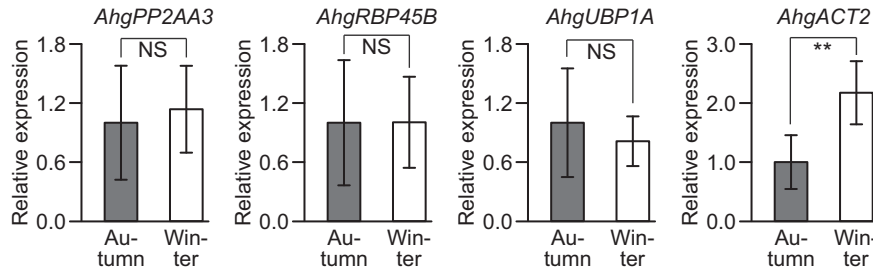


Fig. 2. Expression of ChIP-qPCR reference genes in the field condition. *AhgPP2AA3*, *AhgRBP45B*, *AhgUBP1A* and *AhgACT2* expression was quantified by RT-qPCR using autumn and winter field samples. Expression relative to autumn samples is presented. Values are the means of six biological replicates, and error bars represent standard deviations. Asterisks indicate significance in the Wilcoxon rank sum test: ** $P < 0.01$; NS, not significant at $P < 0.05$.

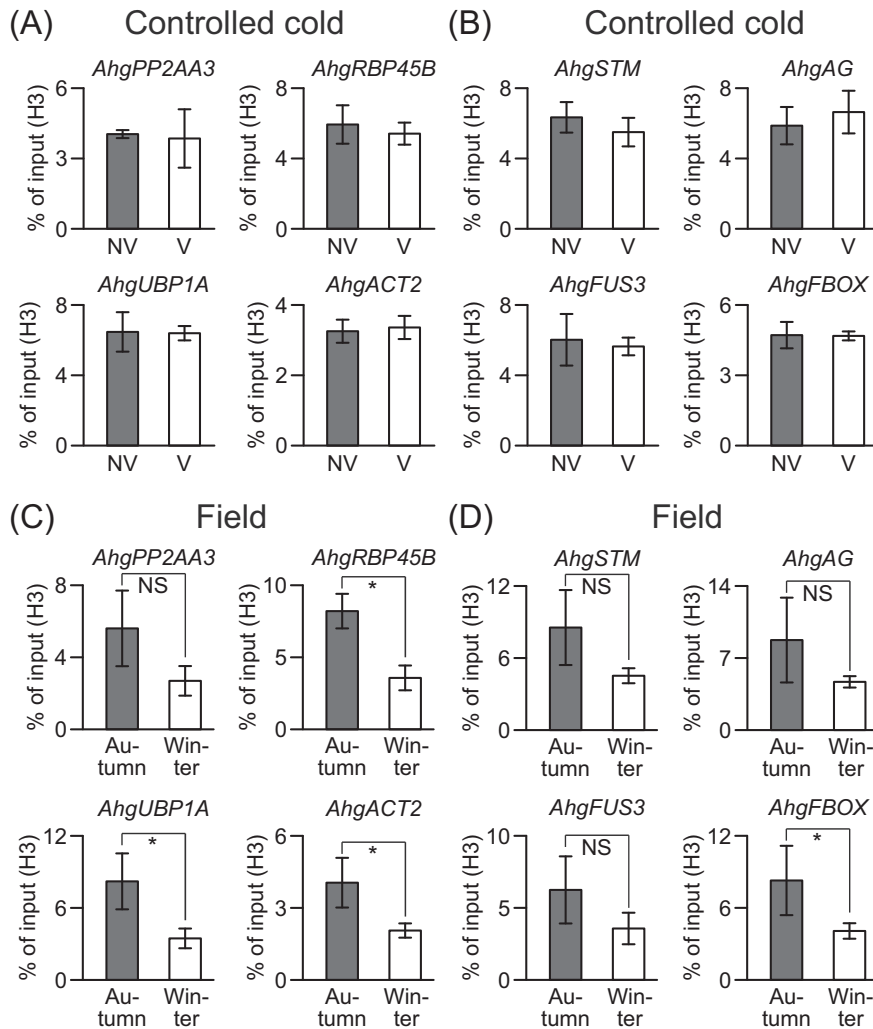


Fig. 3. Amounts of H3 ChIP DNA (% of input) at reference genes in the controlled cold and field conditions. ChIP-qPCR was performed with H3 antibody for candidate reference genes, using non-vernalized (NV) and vernalized (V) laboratory plants (A, B) and autumn and winter plants in the field (C, D). H3 ChIP was performed for *AhgPP2AA3*, *AhgRBP45B*, *AhgUBP1A* and *AhgACT2* (A, C), and for *AhgSTM*, *AhgAG*, *AhgFUS3* and *AhgFBOX* (B, D). Values are the means of three (controlled cold) or four (field) biological replicates, and error bars represent standard deviations. Asterisks indicate significance in the Wilcoxon rank sum test: * $P < 0.05$; NS, not significant at $P < 0.05$.

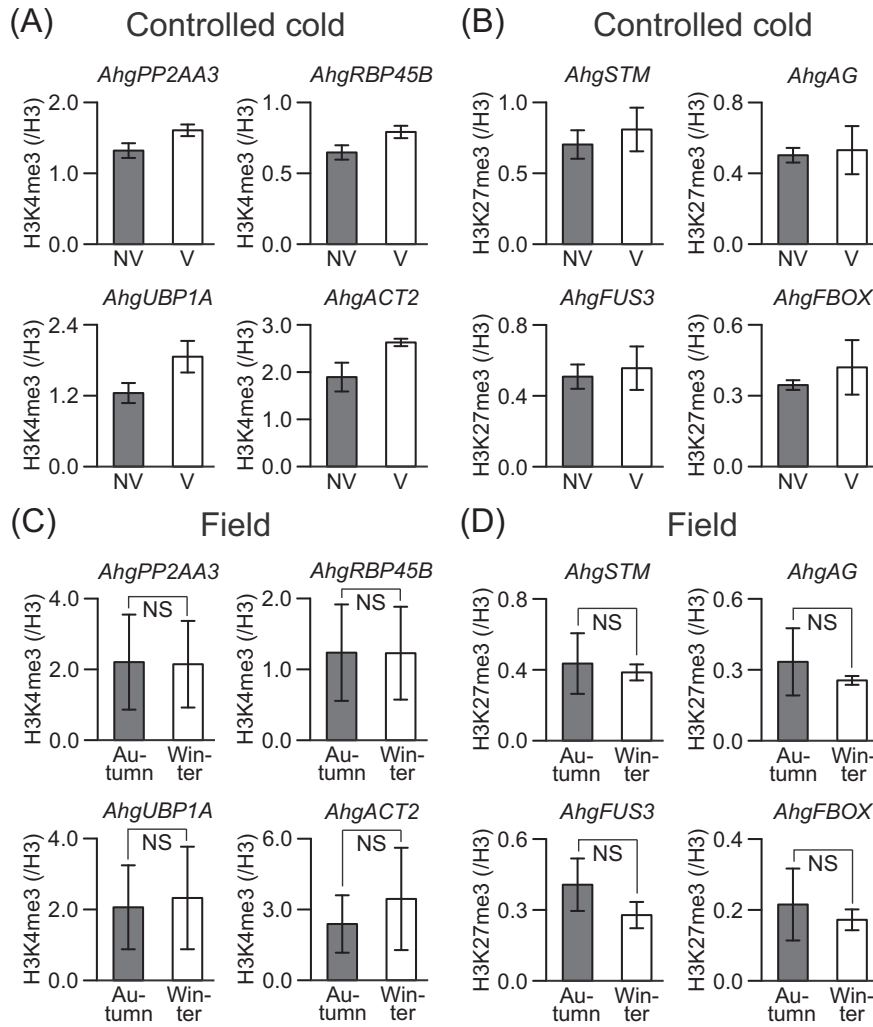


Fig. 4. Validation of ChIP-qPCR reference genes for H3K4me3 and H3K27me3 in the controlled cold and field conditions. ChIP-qPCR was performed for candidate reference genes, using non-vernalized (NV) and vernalized (V) laboratory plants (A, B) and autumn and winter plants in the field (C, D). H3K4me3 ChIP was performed for *AhgPP2AA3*, *AhgRBP45B*, *AhgUBP1A* and *AhgACT2* (A, C), and H3K27me3 ChIP was performed for *AhgSTM*, *AhgAG*, *AhgFUS3* and *AhgFBOX* (B, D). Both H3K4me3 and H3K27me3 levels were normalized by H3 density. Values are the means of three (laboratory) or four (field) biological replicates, and error bars represent standard deviations. NS indicates that the difference is not significant in the Wilcoxon rank sum test at $P < 0.05$.

AhgFLC. H3-normalized values were used for these calculations.

Statistical analyses For the eight reference candidates, Wilcoxon rank sum tests (Wilcoxon, 1945) were performed for gene expression (Fig. 2), absolute amount of H3 ChIP (% input, Fig. 3) and histone modification levels (H3K4me3 and H3K27me3 levels, Fig. 4) to compare autumn and winter samples, for which we had four and six replicates for ChIP and gene expression analyses, respectively, for each sample. Wilcoxon rank sum tests were also applied to compare H3K4me3 and H3K27me3 levels at each region of *AhgFLC* between autumn and winter samples (Fig. 6).

For NV and V, the H3K4me3 and H3K27me3 levels were compared between treatments with different sample preparation (Fig. 5) by two-way ANOVA for repeated measures (Glantz and Slinker, 2000). Effects of the two main factors (treatment and region at the *AhgFLC* locus) were tested at $P < 0.05$ and the interaction was not included.

Note: A detailed procedure for the ChIP steps performed in the field is provided in Supplementary Text S1: “Hands-on protocol for Field ChIP steps”.

RESULTS

Robust ChIP protocols are widely used for the analysis

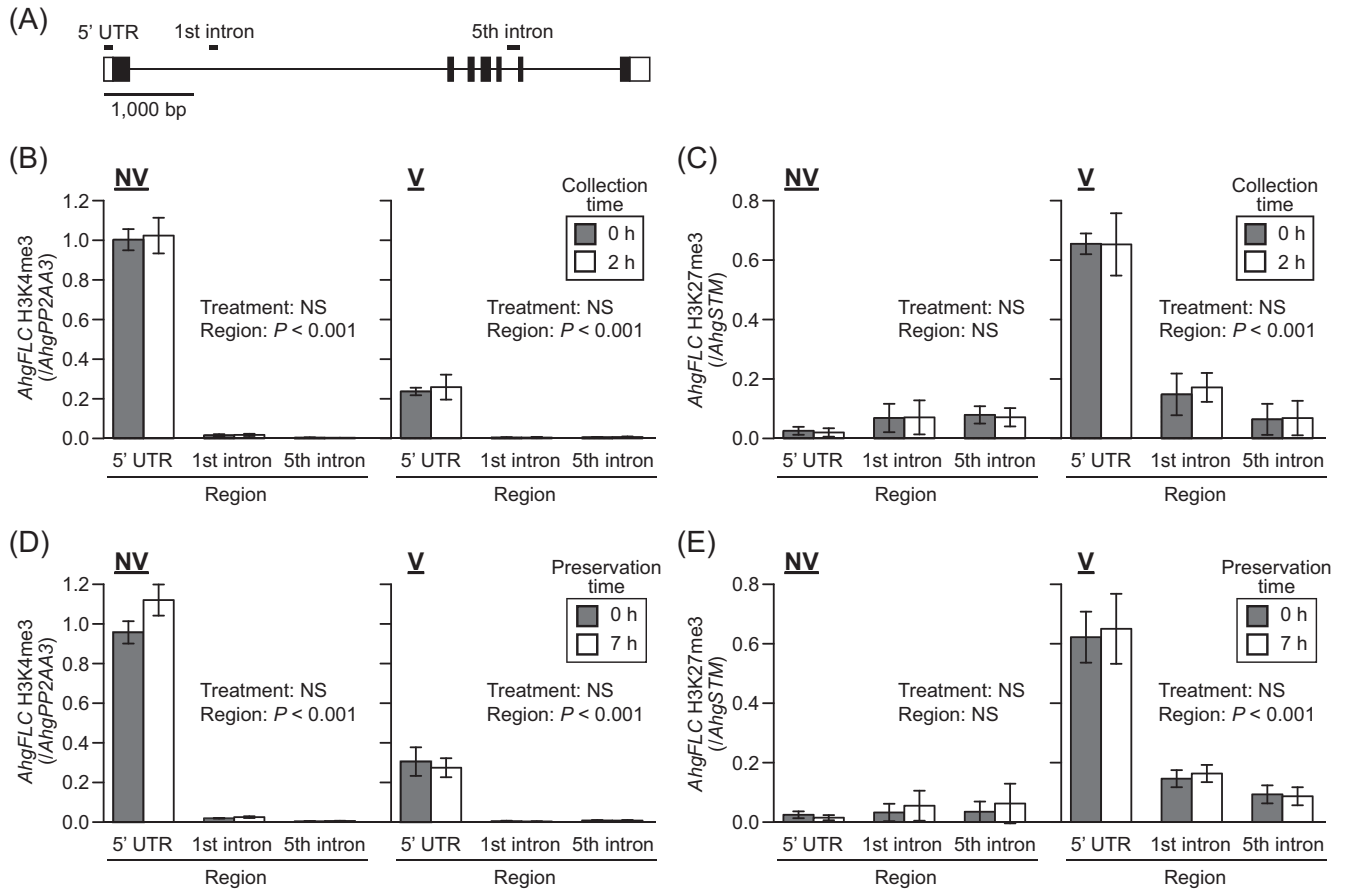


Fig. 5. A schematic diagram of the *AhgFLC* locus showing the three regions in which histone modifications were quantified (A), and the results of modifications of initial steps of ChIP for the field study (B–E). In (A), the diagram is shown in the 5' to 3' direction. Open boxes, filled boxes and solid lines between boxes represent UTRs, exons and introns, respectively. Horizontal bars indicate the three regions where qPCR primers were designed (5' UTR, 1st and 5th intron regions). In (B–E), H3K4me3 (B, D) and H3K27me3 (C, E) levels at the three regions of the *AhgFLC* locus analysed by ChIP-qPCR are shown for two sets of differently treated samples. Grey and white bars in these graphs represent, respectively, 0 h and 2 h sampling time prior to cross-linking (B, C), and 0 h and 7 h preservation time after cross-linking (D, E). The results from non-vernalized (NV) and vernalized (V) plants are shown separately. H3K4me3 levels were normalized to that of *AhgPP2AA3* (B, D). H3K27me3 levels were normalized to that of *AhgSTM* (C, E). Values are the means of three biological replicates, and error bars represent standard deviations. Probability levels for the treatment and region effects of two-way repeated measures ANOVA are listed within each plot. NS indicates non-significance at $P < 0.05$.

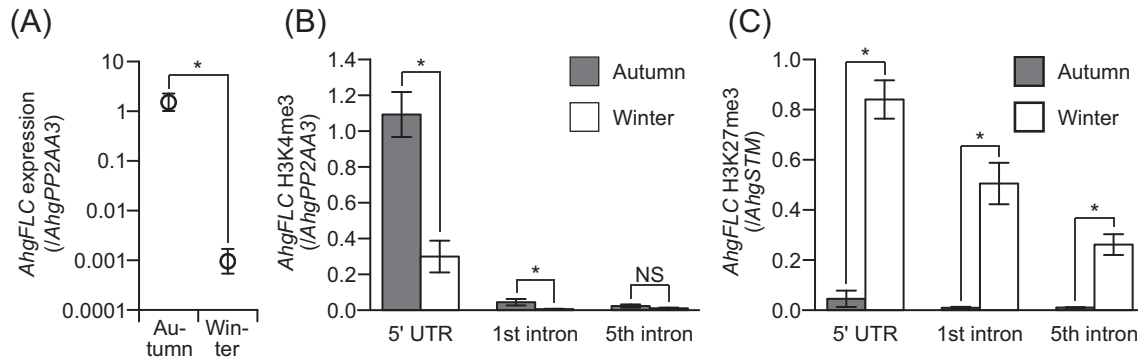


Fig. 6. *AhgFLC* expression and H3K4me3 and H3K27me3 levels at *AhgFLC* in autumn and winter field samples. RT-qPCR was performed to quantify *AhgFLC* expression relative to *AhgPP2AA3*, using autumn and winter field samples (A). ChIP-qPCR was performed for the *AhgFLC* locus (5' UTR, 1st and 5th intron regions) with H3K4me3 (B) and H3K27me3 (C) antibodies using autumn and winter field samples. H3K4me3 levels were normalized to that of *AhgPP2AA3* (B). H3K27me3 levels were normalized to that of *AhgSTM* (C). Values are the means of four biological replicates, and error bars represent standard deviations. Asterisks indicate significance in the Wilcoxon rank sum test: * $P < 0.05$; NS, not significant at $P < 0.05$.

of laboratory-grown samples, especially in species whose genome sequence is available (e.g., Haring et al., 2007; Saleh et al., 2008). We chose to use the protocol described by Gendrel et al. (2005) because it has been widely used for plant samples (e.g., Guo et al., 2008; He et al., 2010; Roudier et al., 2011). We first confirmed that chromatin extracted from cross-linked tissues provided greater enrichment of immunoprecipitated DNA than native chromatin (Supplementary Fig. S2), and confirmed that sonication under our experimental conditions provided DNA fragments with good resolution (around 200–400 bp, Supplementary Fig. S3). We performed chromatin extraction, immunoprecipitation and reverse cross-linking following the protocol of Gendrel et al. (2005).

Identification of ChIP-qPCR reference genes in *A. halleri* and estimation of nucleosome density Reference genes are used to normalize ChIP-qPCR data, i.e., to account for differences in the amount of chromatin between samples. A suitable reference gene must satisfy two major requirements: (1) it should be enriched in the histone modification analysed, and (2) the level of histone modification should remain constant under all experimental conditions (Haring et al., 2007). In the present report, reference genes should not have different histone modification levels between NV and V samples, or between autumn and winter samples.

We selected reference genes among either genes that have been reported to be superior candidates for RT-PCR in *A. thaliana* (Czechowski et al., 2005) or well-characterized genes whose expression was expected not to change in our experimental conditions. As reference genes for H3K4me3 ChIP, we selected orthologues of the following four genes: three genes reported to be active and invariantly expressed under diverse conditions, namely *PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3)*, *RBP45B* and *OLIGOURIDYLATE-BINDING PROTEIN 1A (UBP1A)* (Czechowski et al., 2005), and *ACTIN2 (ACT2)*, a gene constitutively expressed in vegetative tissues (An et al., 1996). As reference genes for H3K27me3 ChIP, we selected orthologues of the following four genes: *SHOOT MERISTEMLESS (STM)* and *AGAMOUS (AG)*, because these genes are silenced in tissues other than the shoot meristem (Yanofsky et al., 1990; Long et al., 1996); *FUSCA3 (FUS3)*, silenced after germination (Gazzarrini et al., 2004); and F-box family protein-related gene (*FBOX*), which has been characterized as a low-expression gene (Czechowski et al., 2005). All of these genes are known to be enriched in H3K27me3 in *A. thaliana* (Zhang et al., 2007) and, moreover, some of them are already routinely used as internal controls (Schubert et al., 2006; Finnegan and Dennis, 2007; Buzas et al., 2011; Lafos et al., 2011). We determined the *A. halleri* sequences of these genes by homology search of *A. thaliana* genes

against the draft genome of *A. halleri* (Supplementary Text S2), and designed the primers for ChIP-qPCR and RT-qPCR (Table 1, Supplementary Fig. S1). We analysed the expression of these candidate reference genes using additional autumn and winter samples of young leaves of *A. halleri* by RT-qPCR. The four candidate reference genes for H3K4me3 ChIP were expressed in both autumn and winter samples (Fig. 2). There was no significant difference ($P > 0.05$, in Wilcoxon rank sum test) in the expression of *AhgPP2AA3*, *AhgRBP45B* and *AhgUBP1A* between autumn and winter, while *AhgACT2* showed 2.2-fold higher expression in winter than that in autumn (Fig. 2). Under our experimental conditions, the expression of four reference genes for H3K27me3 ChIP, i.e., *AhgSTM*, *AhgAG*, *AhgFUS3* and *AhgFBOX*, was undetectable using RT-qPCR.

Nucleosome positioning can vary depending on environmental factors (Huebert et al., 2012), and therefore quantification of H3 tail modifications needs to take into account the density of H3 itself. To approximate H3 density, we quantified the DNA immunoprecipitated with an H3 antibody at all eight putative normalization control genes and compared the quantity between NV and V samples and between autumn and winter samples. For the latter comparison, we had to validate reference genes because the behaviour of internal control genes in ChIP analysis has never been reported for field samples. We therefore analysed the same reference genes used with NV and V samples also for the field samples. Validation of internal controls was based on four biological replicates of the field samples for all ChIPs (H3 ChIP, Fig. 3, C and D; H3K4me3 and H3K27me3 ChIPs, see below and Fig. 4, C and D) and a suitable statistical test (see MATERIALS AND METHODS). Overall, we found little H3 density variation under laboratory-controlled cold conditions (Fig. 3, A and B), and found that differences in H3 density tended to be larger in the field than in the laboratory samples (Fig. 3). The highest change we detected was 2.3-fold, found at *AhgUBP1A* between autumn and winter samples. In the field, one out of the four putative H3K4me3 control genes and three out of the four putative H3K27me3 control genes did not show any significant difference (Wilcoxon rank sum test, Fig. 3, C and D). These results are consistent with the fact that the resolution of our ChIPs is 2–3 nucleosomes. To minimize the influence of histone density on the quantification of histone tail modifications, especially in the field, and for consistency with ChIP-qPCR normalization methods in other reports (Angel et al., 2011; Buzas et al., 2011), we used the H3 ChIP values for normalization in all subsequent calculations.

To assess if these genes satisfy the requirements for reference genes, we first confirmed that they are actually marked with H3K4me3 or H3K27me3. Indeed, all the tested genes showed clearly higher values of % of input

in H3K4me3 or H3K27me3 ChIP than the no-antibody control (Supplementary Table S1). Next, we compared H3K4me3 or H3K27me3 levels at these genes between NV and V, and autumn and winter, also using H3 ChIP as a control for H3 density (see MATERIALS AND METHODS). We found that the H3K4me3 and H3K27me3 levels were similar between NV and V samples for the respective corresponding genes (Fig. 4, A and B). We found no significant differences (Wilcoxon rank sum test) in H3K4me3 or H3K27me3 levels at the regions tested, suggesting that all these regions are suitable normalization controls for autumn and winter field samples as long as they are normalized by H3 density (Fig. 4, C and D). Thus, although all the tested genes were appropriate as reference genes for either H3K4me3 or H3K27me3 ChIP, both in the field condition and in laboratory vernalization, we selected *AhgPP2AA3* and *AhgSTM* as reference genes in which H3 density showed no significant difference between autumn and winter samples (Fig. 3, C and D).

Histone modification patterns at *AhgFLC* during vernalization To characterize histone modifications at *AhgFLC*, we designed primers in three regions along *AhgFLC* corresponding to regions within *A. thaliana FLC* that are known to respond to cold treatment (Finnegan and Dennis, 2007; Angel et al., 2011; Yang et al., 2014, Fig. 5A). We performed H3K4me3 and H3K27me3 ChIPs for the three *AhgFLC* regions, and confirmed that the % of input was much higher in H3K4me3 and H3K27me3 ChIPs than in the no-antibody controls at all three regions (Supplementary Table S2). Next, we compared the levels of histone modifications between NV and V samples. The H3K4me3 level was higher in the NV than in the V condition at the 5' UTR region. The enrichment was low at the 1st and 5th intron regions in both the NV and the V conditions (Fig. 5, B and D). The H3K27me3 level was higher in the V than in the NV condition at the 5' UTR and 1st intron regions but not at the 5th intron region (Fig. 5, C and E). These results indicate that the patterns of both H3K4me3 and H3K27me3 in laboratory-vernalized *A. halleri* are consistent with the reported patterns in *A. thaliana* (Finnegan and Dennis, 2007; Angel et al., 2011; Yang et al., 2014).

Field ChIP: performing the initial steps of laboratory ChIP procedures in the field

1. A long sampling time does not interfere with the results of ChIP-qPCR

Because plants are scattered in their natural habitats, sampling takes a considerably longer time in the field than in the laboratory. In the population of *A. halleri* we are analyzing, it takes approximately 1 to 2 h to collect leaf samples, depending on the number of plants required. To evaluate how such a long sampling time

might influence the results of ChIP-qPCR, we imitated the field sampling by placing leaves harvested in the laboratory in distilled water for 2 h on ice. We then compared the results of ChIP-qPCR for immediately cross-linked (0 h) and 2-h-preserved (2 h) samples using plants grown under the NV or V condition. We found that there was no significant difference between the 0 h and 2 h samples for either H3K4me3 or H3K27me3 ChIP (two-way repeated measures ANOVA, Fig. 5, B and C). These results indicate that long sampling (for up to 2 h) in a natural population allows us to obtain consistent results in ChIP-qPCR.

2. After cross-linking, samples can be preserved for 7 h before deep-freezing

When ChIP is performed in the laboratory, harvested leaf samples are cross-linked with formaldehyde using a vacuum pump and immediately frozen in liquid N₂. We brought a vacuum pump and a rechargeable battery to the field and performed cross-linking at the field site. Since transportation of liquid N₂ to the field would constitute a major traffic hazard, we tested whether samples could be preserved for a 7-h period prior to freezing, representing the longest transport time between our field site and the laboratory. To evaluate whether immediate freezing in liquid N₂ is strictly required, we preserved cross-linked leaves in PBS on ice for 7 h and compared the results of ChIP-qPCR between samples that were immediately frozen in liquid N₂ (0 h preservation time) and those preserved in PBS before freezing in liquid N₂ (7 h preservation time) using plants grown under NV or V conditions. There were no significant differences (two-way repeated measures ANOVA) in the levels of H3K4me3 or H3K27me3 between the 0 h and 7 h samples (Fig. 5, D and E). Thus, preserving cross-linked leaves in PBS on ice for up to at least 7 h after cross-linking allows good preservation of chromatin, comparable to that obtained when tissue is immediately frozen in liquid N₂.

In conclusion, we adapted the Gendrel et al. (2005) standard laboratory ChIP protocol for application to field samples by incorporating two different steps: a 2-h preservation before formaldehyde cross-linking, and a 7-h PBS preservation before freezing in liquid N₂ (see also Supplementary Text S1: "Hands-on Field ChIP protocol").

Histone modifications at *AhgFLC* in autumn and winter are distinct from those in laboratory vernalization

Next, we used the Field ChIP method to sample *A. halleri* leaves in the field site in autumn and winter, and analysed changes in histone modifications at *AhgFLC*. On the same dates, the *AhgFLC* expression level was ca. 1,500 fold higher in autumn than in winter (Fig. 6A). Consistent with this result, the levels of H3K4me3 were higher in autumn than in winter at the 5' UTR and 1st intron regions, but not at the 5th intron

region (Fig. 6B). In contrast, at all of the tested regions, the levels of H3K27me3 were higher in winter than in autumn (Fig. 6C). These results were consistent with those of laboratory vernalization, indicating that the Field ChIP method can reliably detect the critical changes in histone modifications in actual field samples.

At the 1st and 5th intron regions, the increase in H3K27me3 levels upon cold exposure was much more pronounced in the field (Fig. 6C) than in the laboratory (Fig. 5E), while the ratios of winter to autumn (field) and of V to NV (laboratory) for H3K27me3 were similar at the 5' UTR region. The winter/autumn ratio of H3K27me3 at the 1st intron region was greater than 50, and that at the 5th intron region was greater than 20 (Fig. 6C), whereas the V/NV ratios in the laboratory experiment were less than five-fold at the 1st and 5th intron regions (Fig. 5E). This difference was not due to different patterns of H3 density, because they were similar between laboratory and field samples (Supplementary Fig. S4). We found no differences in the levels of H3K4me3 between the laboratory (Fig. 5D) and field (Fig. 6B) samples. Overall, these results indicated that histone modifications at *AhgFLC* are largely similar between laboratory and field samples, but that certain regions of the gene have different patterns.

DISCUSSION

In standard vernalization experiments performed in the laboratory, plants have often been grown under combinations of constant warm and cold temperatures, but such growth conditions do not faithfully mimic natural seasonal environments, in which temperature fluctuations are complex (Kudoh and Nagano, 2013; Kudoh, 2015). While these constant, simplified conditions greatly facilitated the initial dissection of molecular mechanisms of gene expression (e.g., Michaels and Amasino, 1999) and histone modifications (e.g., Bastow et al., 2004; Sung and Amasino, 2004) during vernalization, such simplification may not be informative about molecular mechanisms operating under fluctuating natural environments. Therefore, we took advantage of previously established knowledge about the seasonal pattern of *FLC* expression from a natural population of *A. halleri*, and aimed to extend the analysis to the level of histone modifications.

Characterization of histone modifications has also been initiated in other Brassica species (e.g., Chinese cabbage, Kawanabe et al., 2016). To the best of our knowledge, however, ChIP experiments have not hitherto been performed on field samples through different seasons, and nothing is known about how histone modifications may change in the field. In this report we examined whether a particular set of reference genes are suitable for ChIP-qPCR normalization in *A. halleri*, under both laboratory

vernalization and field conditions. By quantifying both nucleosome density, using H3 ChIP, and H3K4me3/H3K27me3 levels, we demonstrated that these genes are adequate controls, allowing accurate normalization and comparison of H3K4me3 and H3K27me3 levels in the gene of interest at different time points between field and laboratory samples. We also established an effective sampling and preservation method for field samples. Here, we reported the first Field ChIP method applicable to *in natura* plant samples. This method may, furthermore, represent a good starting point for other field studies, provided that prior validation of the suitability of Field ChIP is performed.

Emerging paradigms on how external temperature modulates the developmental transition to flowering have come from studies of *FLC* in *A. thaliana*, in which plants were exposed to controlled temperatures mimicking those in different seasons. These studies pinpointed two main response regions within *FLC* chromatin, the transcription start site and the gene body region (Finnegan and Dennis, 2007; Angel et al., 2011), each associated with quantitative repression of *FLC* by cold and with maintenance of this repression after cold exposure. In this study, we monitored the corresponding regions in a perennial orthologue, *AhgFLC*, and found that the transcription start site registers a decrease in H3K4me3 and an increase in H3K27me3 during winter, similar to that observed in *A. thaliana* upon cold treatment, whereas chromatin responses in the gene body region may be different. H3K27me3 increased considerably at the gene body region during winter, although this increase was less pronounced in laboratory V samples. This is interesting, considering that in *A. thaliana*, the enrichment of H3K27me3 in the *FLC* gene body has been reported to occur after plants return to warm temperature but not in the cold (Finnegan and Dennis, 2007; Angel et al., 2011). The enrichment of H3K27me3 in the *AhgFLC* gene body in winter samples may be explained by longer cold exposure in the field than in the laboratory experiment. Indeed, the calculated cumulative sum of temperatures lower than 10.5 °C, representing the vernalization temperature threshold (Aikawa et al., 2010), was 6,552 degree hours for the laboratory samples and 15,795 degree hours for the field samples. Alternatively, the differences could be caused by other aspects of the natural temperature regimes, such as day-and-night or day-by-day fluctuations of temperature and a gradual change of mean temperature in the long term. The significance of these findings should be substantiated by more detailed future studies in which the laboratory experimental protocol is adjusted to simulate the actual *in natura* conditions. With the availability of our current Field ChIP method, and using *FLC* as a model system, such studies are now possible and should greatly expand our understanding of chromatin's contribution to

the regulation of gene expression *in natura*.

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