Robust control of the seasonal expression of the *Arabidopsis FLC* gene in a fluctuating environment

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Plants flower in particular seasons even in natural, fluctuating environments. The molecular basis of temperature-dependent flowering-time regulation has been extensively studied, but little is known about how gene expression is controlled in natural environments. Without a memory of past temperatures, it would be difficult for plants to detect seasons in natural, noisy environments because temperature changes occurring within a few weeks are often inconsistent with seasonal trends. Our 2-y census of the expression of a temperature-dependent flowering-time gene, *AhgFLC*, in a natural population of perennial *Arabidopsis halleri* revealed that the regulatory system of this flowering-time gene extracts seasonal cues as if it memorizes temperatures over the past 6 wk. Time-series analysis revealed that as much as 83% of the variation in the *AhgFLC* expression is explained solely by the temperature for the previous 6 wk, but not by the temperatures over shorter or longer periods. The accuracy of our model in predicting the gene expression pattern under contrasting temperature regimes in the transplant experiments indicates that such modeling incorporating the molecular bases of flowering-time regulation will contribute to predicting plant responses to future climate changes.

Arabidopsis halleri | *FLOWERING LOCUS C* | gene regulation | natural temperature fluctuation | plant phenology modeling

A unique paradigm that has arisen from a systems biological approach is that gene regulation under a fluctuating environment is significantly different to that seen under constant laboratory conditions (1). This was recently shown for the flowering phenotypes of *Arabidopsis* mutants grown in natural environments (2). Many genes are involved in flowering-time control (3–5), but little is known about how these genes function in nature (1). Plants flower at particular times of the year using environmental cues to detect seasons (6), and flowering with other timing often decreases reproductive success substantially (7, 8). Because genetic systems, such as flowering time control, operate in natural habitats, a full understanding of gene function requires the study of gene regulation in complex environments.

Temperature is a major seasonal cue by which plants determine when to flower. In natural conditions, temperature shows day/night, day-by-day, and weekly fluctuations that are often inconsistent with the seasonal trends. Plants, however, control flowering time precisely even in the noisy natural environment. We expected, therefore, that plants would have a gene regulatory system that is characterized by a memory of past temperatures, and this memory should be long enough to buffer shorter-term temperature fluctuations, but short enough to be sensitive to the seasonal temperature trend.

In the MADS-box gene family of *Arabidopsis thaliana*, genes belonging to a monophyletic clade, including the *FLOWERING LOCUS C* (*FLC*) and *MADS AFFECTING FLOWERING* (*MAF*) gene subfamily, have been found to encode transcription factors that regulate flowering time in response to temperature change (9). *FLC* represses flowering by down-regulating the floral pathway integrators, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) (10, 11). The *FLC* expression is down-regulated in response to prolonged cold (vernalization), and this response ensures that flowering occurs after winter (12–14). *MAF2* is probably involved in the vernalization response to short periods of cold within 3 wk, and the role of other *MAF* genes remains to be determined (15). In the perennial life cycle, *PERPETUAL FLOWERING 1* (*PEP1*), an *FLC* ortholog of *Arabis alpina*, controls flowering transition by being down-regulated in response to prolonged cold, and the reverse transition by being up-regulated in response to a warm temperature after cold (16). We expected that the gene regulation of *FLC* orthologs may serve as the mechanism to extract seasonal cues from natural environments, because they are regulated by histone modification (17–19), which is often involved in stable cellular memory (20, 21).

Herein we report on a 2-y census on the gene expression of an *FLC* ortholog *AhgFLC* in a natural population of perennial *Arabidopsis halleri* subsp. *gemmifera* (referred as to *A. halleri* hereafter) in central Honshu, Japan (Fig. S1). The aim of this study was to evaluate how *AhgFLC* is regulated during natural temperature fluctuations—specifically, for how long the gene regulatory system holds the memory of past temperatures to detect seasons.

Results

Characterization of *AhgFLC*. An *AhgFLC* full-length cDNA sequence showed 95.9% and 94.9% identity in nucleotide and amino acid sequences, respectively, with *A. thaliana FLC* (Fig. S2, Table S1). *AhgFLC* formed a clade with *A. thaliana FLC* and other *FLC* orthologs of related Brassicaceae in a neighbor-joining cladogram, and *A. thaliana MAF* genes formed a distinct clade (Fig. 1*A* and *SI Materials and Methods*). *Arabidopsis halleri* showed vernalization responses to 4 wk or longer cold treatment, which promoted flowering and suppressed the *AhgFLC* expression (Fig. S3). Furthermore, a strong delay in flowering was observed in transgenic *A. thaliana* in which *AhgFLC* was constitutively expressed (Fig. 1 *B* and *C*). These findings suggest that *AhgFLC* is an ortholog of *FLC*.

Seasonal Pattern of *AhgFLC* Expression in a Natural Environment. We quantified the expression of *AhgFLC* in a natural population of *A. halleri* for ~2 y by visiting the field site 96 times at ~1-wk intervals. At every visit, the *AhgFLC* expression level in the leaves of six plants was measured by real-time quantitative PCR and then quantified relative to the level for a standard sample obtained

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Data deposition: The cDNA sequences reported in this paper, AhgFLC, AhgFT, AhgAP1, AhgSOC1, AhgLHP1, AhgVIN3, and AhgVRN2 have been deposited in the GenBank database (accession nos. AB465585, AB465586, AB465587, AB465588, AB465589, AB465590, and AB465591, respectively).

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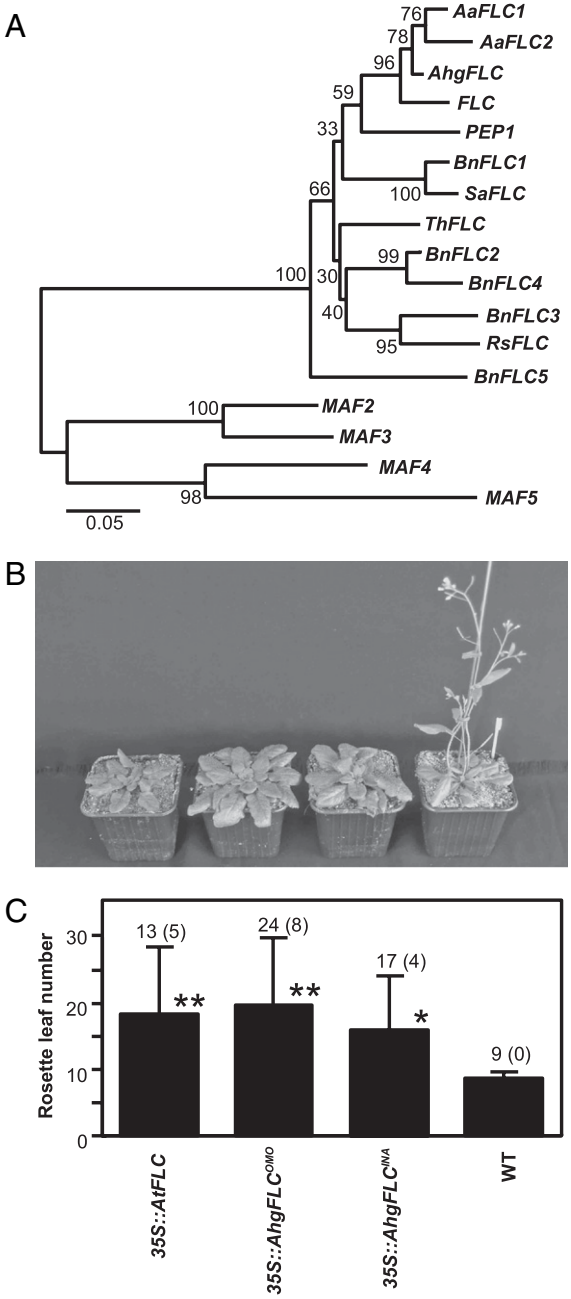

Fig. 1. Sequence analysis and transformation experiments of *AhgFLC*. (*A*) *AhgFLC* forms a clade with *A. thaliana FLC* (*FLC*) in a neighbor-joining cladogram including *FLC* homologs of related plants in Brassicaceae. *AaFLC*, *BnFLC*, *RsFLC*, *SaFLC*, *ThFLC*, and *PEP1* represent *Arabidopsis arenosa*, *Brassica napus*, *Raphanus sativus*, *Sinapis alba*, *Thellungiella halophila*, and *Arabis alpina* homologs of *FLC*, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above nodes, and branch lengths are proportional to the nucleotide substitution rate. (*B*) Strong delay in flowering was observed for transgenic plants in which *AhgFLC* was constitutively expressed in *A. thaliana*. Phenotypes of 7-wk-old transgenic T1 *A. thaliana* (Col) expressing *FLC* and *AhgFLC* are shown from left to right: *35S::FLC*^{Col}, *35S::AhgFLC*^{OMO}, *35S::AhgFLC*^{INA}, and nontransgenic wild-type plant. *AhgFLC*^{OMO} and *AhgFLC*^{INA} were obtained from different individuals of *A. halleri*. (*C*) Number of rosette leaves of the transgenic plants and wild type at flowering (mean and SD). The numbers above the bars are the total number of plants tested together with the number of nonflowered plants in parentheses. The number of leaves of nonflowered plants was set as 30 in the mean calculation because all nonflowered plants had more than 30 leaves at the end of the growth experiments. Asterisks indicate significant differences in the number of leaves as compared with that of the wild type (Wilcoxon rank-sum test; *$P < 0.05$; **$P < 0.001$).

from a 6-wk cold-treated plant. The *AhgFLC* expression showed a clear seasonal pattern in both years (Fig. 2*A*). We observed strong winter repression, with the average levels of maximally suppressed *AhgFLC* being 1–3% of those of the 6-wk cold-treated standard and less than 0.03% of those of the yearly maximum (Fig. 2*A*). Notably, the decreases and increases of *AhgFLC* expression occurred slowly over multiple weeks following the long-term seasonal trends of temperature change. The diurnal variation of *AhgFLC* expression was much smaller compared with the seasonal variation, and the minimum expression levels within 48 h were about 20% of the maximum values for the two measurement periods at the vernal and autumnal equinoxes (Fig. S4).

Phenological transitions corresponded with the expression levels of *AhgFLC* (Fig. 2 *A* and *B*). We recorded the timing of bolting (elongation of the flowering stem, the earliest morphological sign of flowering) and flower opening of 160 naturally growing plants. We also observed the reverse transition from reproductive to vegetative growth by recording the timing of inflorescence reversion, in which leaf formation occurs at the apical meristem after inflorescence development (Fig. 2*C*). The average *AhgFLC* expression levels at the peaks of bolting were low, 0.085 and 0.071 in 2007 and 2008, respectively, and they remained low until the end of March in both years (Fig. 2 *A* and *B*). By the beginning of the reverse transition to vegetative growth, the *AhgFLC* expression levels increased to nearly the prewinter levels (Fig. 2 *A* and *B*). The gross morphology of meristems in the phenology observations corresponded with the expression of *AhgAP1*, a homolog of the reporter gene for identifying floral meristem *APETALA1* (22). The expression of *AhgAP1* was observed at meristem at least during bolting and flowering, and the timing of its suppression corresponded with that of reverse transition (Fig. 2*D*).

Time-Series Analyses of the *AhgFLC* Expression. We applied three chilling-unit models that are commonly used to predict plant phenology (23) to analyze the relationship between the *AhgFLC* expression and temperature in the natural habitat. First, we developed a chilling accumulation model in which the *AhgFLC* expression levels are assumed to be determined by the cumulative sum of temperatures lower than a certain threshold (T) over a past given period (L). Using the maximum likelihood method, we estimated L and T (Fig. 3*A*). The results showed that the level of the *AhgFLC* expression was best explained by the temperature regime over the previous 42 d [95% confidence interval (CI): 39–45 d], with a threshold temperature of 10.5 °C (95% CI: 9.7 °C–11.1 °C). Surprisingly, the proposed model explained as much as 83% of the variation in the *AhgFLC* expression level (Fig. 3*B*). The log likelihood was more sensitive to the change in L than to that of T (Fig. 3*A*), implying that the length of the period during which past temperature was assumed to determine the current *AhgFLC* expression was a more influential factor. We also tested two other models, chilling hour and exponential decay models. Instead of cumulative sum of temperature below the threshold, the chilling-hour model used the cumulative sum of hours, and the exponential decay model incorporated a decay factor by which the effect of past temperature was diminished. All three models explained ≈80% of the variation in *AhgFLC* expression solely by temperature if we assume that the expression level is determined by the temperature of the past ~6 wk (Table S2). The analyses using the hourly air temperature at the nearest meteorological station and the hourly ground-surface temperatures at the study site showed similar results (Table S2).

Transplant Experiments with Controlled Temperature Conditions. To evaluate the predictions of the model, we conducted a set of transplant experiments and compared the predicted and observed *AhgFLC* expression levels. Field-grown plants were transplanted into two laboratory controlled conditions, i.e., 20 °C/15 °C and 4 °C/4 °C

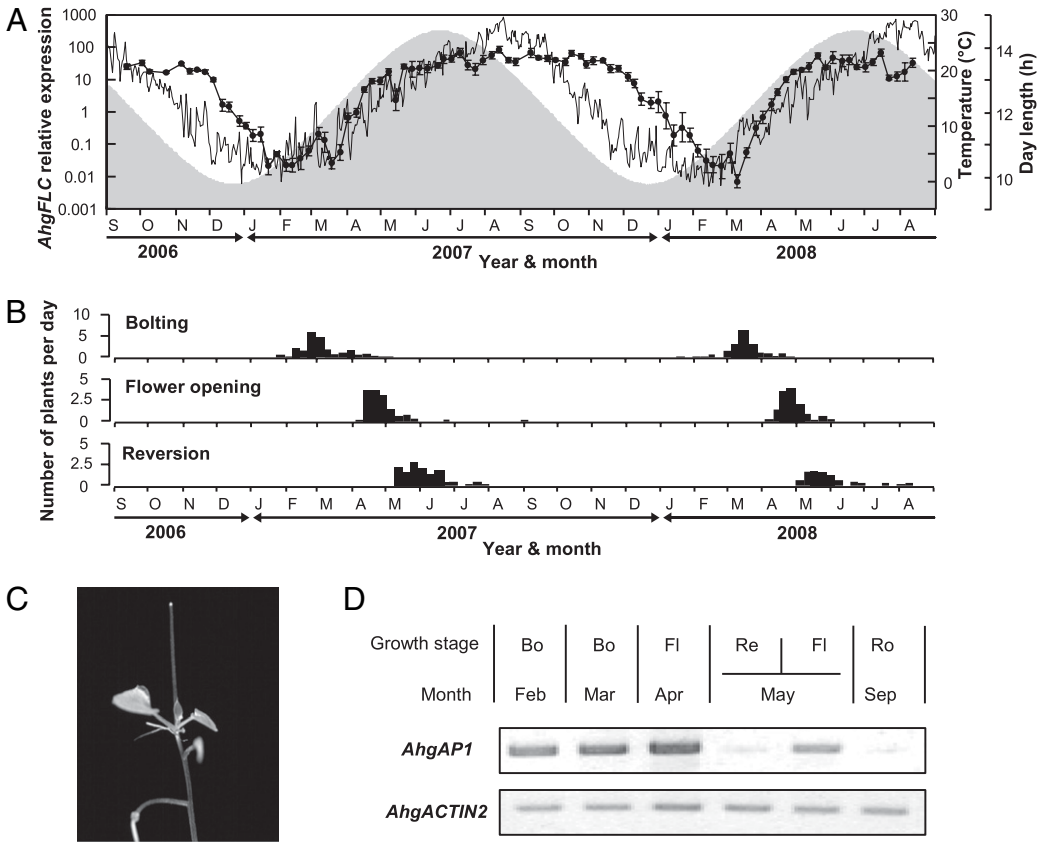

Fig. 2. Expression of *AhgFLC* in the natural habitat shows a seasonal pattern that corresponds with plant phenology. (*A*) Seasonal pattern of the *AhgFLC* expression in leaves (solid line and circles, mean ± SD, $n = 6$) measured at ~1-wk intervals for 2 y. The thin line indicates daily mean air temperature, and photoperiods are shown by the shading. (*B*) Phenology of the study population. The number of plants per day that initiated bolting, flower opening, and inflorescence reversion, respectively, between the two successive censuses are shown. (*C*) Infloresence reversion, i.e., leaf formation at the reproductive shoot-apical meristem. (*D*) The expression of *AhgAP1* in shoot apical meristems corresponded to plant phenology. The *AhgAP1* was expressed throughout the period from bolting to flower opening in the apical meristems [left three lanes; meristem tissues of plants at bolting (Bo) in February and March, and at flowering (Fl) in April]. In May, *AhgAP1* RNA was present in the meristems of flower-producing inflorescences but absent in the meristems of reverted inflorescences [fourth and fifth lanes; meristem tissues at inflorescence reversion (Re) and flowering (Fl) in May, respectively]. The expression of *AhgAP1* was not detected in the meristem of vegetative rosettes [sixth lane; meristem tissue at the rosette stage (Ro) in September]. *AhgACTIN2* was used as a loading control. The GenBank accession number for the *AhgAP1* cDNA sequence is AB465587.

(day/night temperature for a 12-h day length), and the temporal changes in the *AhgFLC* expression were measured for the next 3 mo. When plants were transplanted before the decrease in the *AhgFLC* expression (November 12, 2007), the *AhgFLC* expression level in the transplants gradually decreased at 4 °C/4 °C but remained high at 20 °C/15 °C (Fig. 4*A*). When plants were transplanted before the increase in the *AhgFLC* expression (February 19, 2007), the *AhgFLC* expression level in the transplants gradually increased at 20 °C/15 °C but remained to be suppressed at 4 °C/4 °C (Fig. 4*B*). The observed changes in the *AhgFLC* expression level were mostly consistent with the model predictions, but the decrease of the *AhgFLC* expression continued for ~9 wk in the November transplants (Fig. 4 *A* and *B*).

Semiquantitative Reverse-Transcription PCR for Putative Vernalization Genes. The seasonal expression was further examined for genes homologous to those involved in the vernalization response of *A. thaliana*. The seasonal changes of expression of these genes occurred during winter and successive flowering periods, and the expression states after the reverse transition returned to the pre-winter state (Fig. 5). The expression of *AhgSOC1*, a homolog of *FLC*-downstream flowering activator, corresponded quantitatively with the repression of *AghFLC* (Fig. 5). Although weak signals of *AhgFT*, another homolog of *FLC*-downstream flowering activator, were present in February and March, a strong expression was observed after the photoperiods became longer in April (Fig. 5). Thereafter, the *AhgFT* expression was repressed and corresponded with a high expression of *AhgFLC* (Fig. 5). *AhgVIN3*, a homolog of the *VERNALIZATION INSENSITIVE3* (*VIN3*) required for the initial repression of *FLC* (24), was expressed during the months of low temperatures, and a high expression corresponded with the period of continual decline in the *AhgFLC* expression (Fig. 5). *AhgLHP1*, a homolog of *LIKE HETEROCHROMATIN PROTEIN1* (*LHP1*) required for the maintenance of the *FLC* repression (25, 26), was expressed year-round. An increase of *AhgVRN2*, a homolog of *VERNALIZATION 2* (*VRN2*) whose product is known to constitutively associate with the H3K27me3 sites of the *FLC* locus (17, 19, 27), was observed corresponding with the repression of *AhgFLC* (Fig. 5).

Discussion

Our findings suggest that *Arabidopsis halleri* plants detect the seasonal trend of temperature at the level of gene regulation. The memory and buffering functions of *AhgFLC* that were revealed to last for ~6 wk are likely to act as a filter to extract the seasonal cue from the noisy natural environment. *FLC* is regulated by PcG protein-mediated gene repression through histone modification (17–19), which is known to be a stable gene regulation involved in

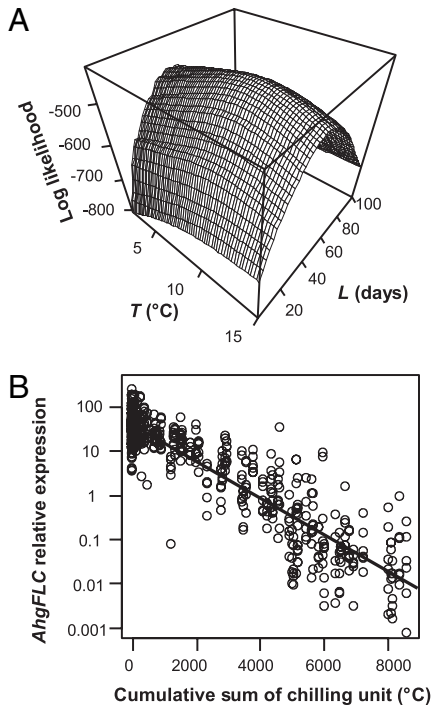

Fig. 3. Time-series analyses revealed that seasonal variation in the *AhgFLC* expression is mostly explained by temperature during the past ~6 wk. (*A*) 3D surface plot of log likelihood function of *T* (base temperature) and *L* (length of period) in the chilling accumulation model. As indicated by the peak of the surface (the maximum likelihood), the level of *AhgFLC* was best explained by the temperature regime over the past 42 d with a base temperature of 10.5 °C. (*B*) The relative expression of *AhgFLC* as a function of the cumulative sum of chilling units with the maximum likelihood estimates (L = 42 d and T = 10.5 °C). The model explained most of the variation of *AhgFLC* (adjusted R^2 = 0.83). Log-transformed variables were used for the relative expression of *AhgFLC*. The line represents the linear regression, which has an intercept of 1.58 (SE = 2.8×10^{-2}, $P < 0.001$) and a slope of -4.1×10^{-4} (SE = 8.0×10^{-6}, $P < 0.001$).

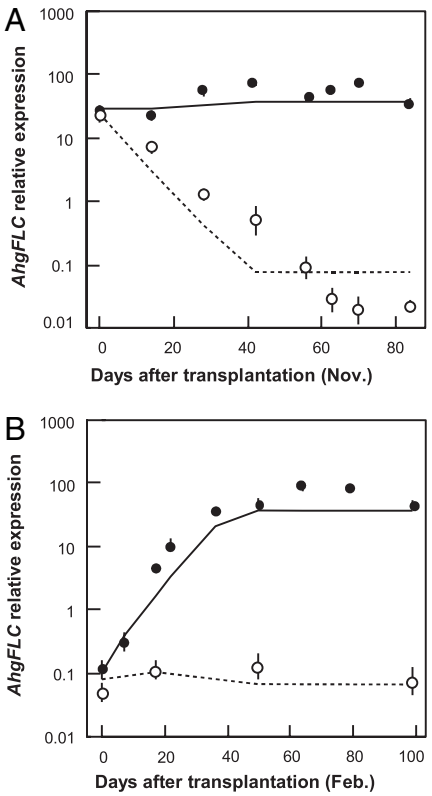

Fig. 4. Changes in *AhgFLC* expression in the transplant experiments and the model predictions. Plants with high (*A*) and low (*B*) *AhgFLC* expression were transplanted into the two controlled conditions (20 °C/15 °C and 4 °C/4 °C, day/night temperatures) in November and February, respectively. *AhgFLC* expressions (mean ± SD, n = 6) are shown by closed and open circles for the 20 °C/15 °C and 4 °C/4 °C conditions, respectively. The predictions based on the chilling accumulation model are shown by solid and dashed lines for 20 °C/15 °C and 4 °C/4 °C, respectively.

many kinds of cellular memory (20, 21). Repression of the *FLC* is stable after cold in *A. thaliana* (14, 28), and this is shown to be maintained at the *FLC* locus by H3K27me3, a universal repressive histone mark (19). *PEP1*, an *FLC* ortholog of perennial *Arabis alpina*, is up-regulated in response to warm temperature, and H3K27me3 at *PEP1* is not maintained after cold (16). These mechanisms for the *FLC* and *PEP1* regulations led us to expect that a similar mechanism might provide the functions of *AhgFLC* as a memory and a buffer.

The natural level of the *AhgFLC* expression was strongly correlated with the seasonal trend of past temperatures in a dosage-dependent manner. We therefore consider that *AhgFLC* provides not merely a memory of winter but a quantitative memory throughout the year. Although the underlying mechanism is currently unknown, the involvement of a quantitative regulation throughout a relative balance of repressive and active histone marks has been suggested by the *A. thaliana FLC* regulation (29, 30). Our model analyses estimated that temperatures during the previous ~6 wk determined the level of *AhgFLC* expression, but down-regulation of *AhgFLC* continued over 6 wk when plants were moved to cold temperatures in November in the transplant experiments. This may be an indication that periods of past temperature that affect the *AhgFLC* expression is longer in the down-regulation in comparison with the up-regulation processes. The additional analyses using half-year data during up- and down-regulation periods of *AhgFLC* estimated L as ~20 and 60 d, respectively, although these models did not improve the predictions for the transplant experiment results (Table S2).

The *AhgFLC* expression level in natural conditions was correlated not only with bolting time, but also with the timing of inflorescence reversion. The seasonal regulation of *AhgFLC* is therefore likely to serve as a robust mechanism that controls both the initiation and termination of flowering in the noisy natural environment. The results of *AhgFLC* characterization suggest that *AhgFLC* is the flowering repressor of *A. halleri*, but to fully substantiate this we require further genetic or transgenic evidence

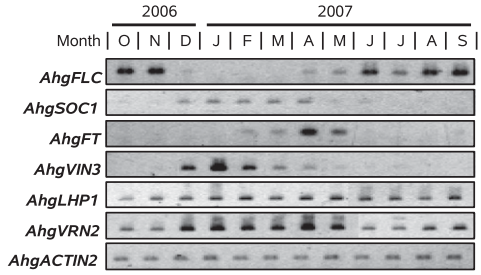

Fig. 5. Semiquantitative RT-PCR analyses of seasonal expression patterns of *AhgFLC*, *AhgSOC1*, *AhgFT*, *AhgVIN3*, *AhgLHP1*, and *AhgVRN2* in leaves. These genes are *A. halleri* homologs (95% or greater sequence homology) of the corresponding *A. thaliana* genes, which are involved in the vernalization response. The GenBank accession numbers for the *AhgFLC*, *AhgSOC1*, *AhgFT*, *AhgVIN3*, *AhgLHP1*, and *AhgVRN2* cDNA sequences are AB465585, AB465588, AB465586, AB465590, AB465589, and AB465591, respectively.

from the study species. *PEP1* has been genetically proven to limit the duration of perennial *Arabis alpina* flowering and facilitates a return to vegetative growth (16). Although reversible regulation of flowering repressors is a necessity for perennial life-cycles (16), it means that the warm temperature required for reproductive growth after cold also up-regulates the flowering repressor. In *A. halleri*, the combination of strong winter repression and a gradual increment in the *AhgFLC* expression are likely to provide the time window during which flowering takes place. At the lowest level, the natural *AhgFLC* expression was repressed much more markedly than in the standard plant that was experimentally treated for 6 wk in the cold. The situation is in contrast with that of *A. thaliana FLC*, in which stable repression after cold allowed flowering to occur in successive warm temperatures (14, 28). However, some northern lines are known to show reversibility in the *FLC* repression (29).

The putative homologs of downstream genes also showed seasonal patterns that corresponded to the flowering phenology of *A. halleri.* In natural conditions, repression of *AhgFLC* and the rise of *AhgSOC1* start in December under the shortest day-lengths, followed by *AhgAP1* expression at the meristem and bolting in February and March. Weak *AhgFT* signals were present at bolting, although the strong *AhgFT* expression occurred much later, corresponding with a 12-h or longer day-length in April. The *AhgFT* expression ceased in late May, corresponding with the inflorescence reversion and a spring increase of *AhgFLC*, notwithstanding that the plants were subjected to the longest photoperiods. In *A. thaliana*, *FLC* acts as an inhibitor of *SOC1* and *FT* (10, 11), but the repression of *FLC* is not sufficient to activate *SOC1* and *FT* (31, 32). *SOC1* is activated by a gibberellin-dependent pathway under a short day (32) and by a photoperiod-dependent pathway under a long day (33, 34). *FT* activation depends strongly on the photoperiod (31). The observed expression patterns of *A. halleri* homologs may represent the temporal shift of relative importance of different pathways in controlling flowering phenology under seasonal environments.

In the phenological modeling of plants, a "chilling requirement" is often incorporated to predict flowering and budburst (23). A major problem with these models has been the lack of direct measurement of the developmental state of plants during the cold (23). Our study indicates that gene expression data can serve as such measurements and give a mechanistic explanation as to why these models predict plant phenology. Although our study is one of few examples of the measurement of temporal changes in gene expression under natural conditions (1), such approaches will become increasingly important in the prediction of plant responses to future climate change in diverse ecosystems.

Materials and Methods

Some details not included here, as well as primer information (Table S1), appear in *SI Materials and Methods*.

Study Site. The plant population (35°06′ N, 134°55′ E, altitude 190–230 m) occurs on an open site alongside a small stream that runs through secondary forest (Fig. S1). Thousands of *A. halleri* plants continually grow in a habitat that extends for ~400 m along the stream. At the study site, we monitored the *AhgFLC* expression, plant phenology, and the ground-surface temperatures during the study period. Temperature records were also obtained from the nearest meteorological station (Nishiwaki, 35°00′N, 134°59.9′E, altitude 72 m, Japan Meteorological Agency). Average temperatures during the study period (n = 19,464) were 15.77 °C and 15.57 °C for the hourly ground-surface temperature at the study site and the hourly air temperature at the meteorological station, respectively, and Pearson's correlation coefficient between them was 0.966.

***AhgFLC* Expression Quantification.** In a natural population of *A. halleri*, we quantified the expression of *AhgFLC* for ~2 y from September 19, 2006, to August 11, 2008, at ~1-wk intervals. Six large plants with multiple clonal rosettes were selected to conduct multiple sampling on the same individuals. A young leaf that reached to ~10 mm in length was collected from each plant on the sampling date. Total RNA from 5 to 10 mg of leaf tissue was extracted using an RNeasy Plant Mini Kit (Qiagen). The *AhgFLC* expression was quantified by 7300 Real-Time PCR System (Applied Biosystems).

Time-Series Analyses. We developed three chilling unit (CU) models to explain $y(t)$, log (*AhgFLC* expression level) at time t, based on $x(t)$, a function of the past temperature regime designated in each model. In the chilling accumulation model, a CU is defined as $CU(t) = \begin{cases} T - s(t) & \text{if } s(t) \le T \\ 0 & \text{otherwise} \end{cases}$, where T is base (threshold) temperature and $s(t)$ is temperature during hour t. $y(t)$ is assumed to depend on the cumulative sum of CU over a certain period (L days) before the measurement, $x(t) = \sum_{n=0}^{24L-1} CU(t-n)$. $y(t)$ was regressed using a linear function of $ax(t) + b$, where a and b are regression coefficients.

In the chilling-hour model, a chilling unit is simply defined as $CU(t) = \begin{cases} 1 & \text{if } s(t) \le T \\ 0 & \text{otherwise} \end{cases}$. This model assumes that the effect of temperature is proportional only to the time period as long as it is lower than the threshold.

In the exponential decay model, the definition of *CU* was same with that in the chilling accumulation model, but $x(t)$ was calculated as $x(t) = \sum_{n=0}^{\infty} e^{-\alpha(t-n)} CU(t-n)$. This model assumes that $y(t)$ is influenced by temperature in the recent past with a decay parameter, α. The confidence interval of the parameters was calculated by the bootstrapping method. For these analyses, the data from the six individuals were pooled.

Transplant Experiments. Six plants from the field site were transferred to each of the 20 °C/15 °C and the 4 °C conditions. *AhgFLC* was quantified just before the transplantation and periodically at 7- to 39-d intervals for ~3 mo after the transplantation. Predictions from the chilling accumulation model (L = 42, T = 10.5) were calculated based on the temperatures experienced by the transplants.

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