Supporting Information

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SI Materials and Methods

Study Species and Site. *Arabidopsis halleri* (L.) O'Kane and Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane and Al-Shehbaz is distributed in the Russian Far East and East Asia (1). The study site is located alongside an upstream section of the Omoide-gawa River, Makino, Naka-ku, Taka-cho, Taka-gun, Hyogo Prefecture, Japan (Fig. S1). *Athyrium yokoscense* (Franch. and Sav.) Christ and *Miscanthus sinensis* Anderss. are common species at the study site. The hourly ground-surface temperature was recorded using a data logger with a thermal sensor (Hobo Water Temp Pro; Onset) set in the soil near to the ground surface. The instrument was operating from 98 d before the first *AhgFLC* measurement to the end of the experiment period.

Sequence Analyses. cDNA sequences of *AhgFLC* were determined using the Sanger method. Full-length *AhgFLC* cDNA was amplified using primers from the 5′UTR (untranslated region) and 3′UTR (Table S1). PCR products were directly sequenced using an ABI PRISM 3100 Genetic Analyzer. A gene phylogeny tree was generated by the neighbor-joining method as implemented in MEGA4 with 1,000 bootstrap replicates (2). Full-length sequences of *FLC* homologs of Brassicaceae and *MAF* gene family members of *A. thaliana* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Sequin/acc.html) and aligned by ClustalW (http://clustalw.ddbj.nig.ac.jp/top-e.html) and manually corrected. The included sequences were: *Arabidopsis thaliana FLC* (*FLC*; AF537203), *A. arenosa FLC* (*AaFLC1*, *2*; DQ167446, DQ167444), *Brassica napus FLC* (*BnFLC1*, *2*, *3*, *4*, *5*; AY036888, AY036889, AY036890, AY036891, AY036892), *Raphanus sativus FLC* (*RsFLC*; AY273160), *Sinapis alba FLC* (*SaFLC*; EF542803), *Thellungiella halophila FLC* (*ThFLC*; AY957537), *Arabis alpina PEP1* (*PEP1*; FJ755930), *MAF2* (AY231441), *MAF3* (AY231445), *MAF4* (AY231450), and *MAF5* (AY231455). The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 585 positions in the final dataset used for the analysis. Tandem or small-scale segmental duplications are recently reported from *Arabidopsis* relatives, in which *A. thaliana* has one copy and its relatives have a few orthologous copies (3), e.g., two full-length *FLC* copies in *A. arenosa* (4, 5). The presented tree is intended to show that *AhgFLC* is in the clade of *FLC* homologs *sensu stricto* such that is distinct from the *MAF* gene subfamily clade. Because recombination/gene conversion was detected among *Arabidopsis FLC* homologs [four-gamete test implemented in DnaSP (6), http://www.ub.edu/dnasp/], the relationship between these homologs may differ in different regions. Primer sequences for real-time quantitative PCR used in this study are conserved among *AhgFLC*, *FLC*, and *AaFLC1* and *2*.

Vernalization Response. We examined the responses to prolonged cold of the flowering and *AhgFLC* expression. Four sets of 12 plants were grown at 20 °C/15 °C for 8 wk after germination. Thereafter, each of four sets of plants was placed at 4 °C for 0, 1, 4, or 8 wk (see *Growth Experiment* for detailed growth conditions). The *AhgFLC* expression was quantified for six plants for each treatment immediately after the cold treatment and compared with that in the 0-wk cold control. After the cold treatment, plants were placed at 20 °C/15 °C, and the numbers of bolted and flowered plants were recorded at 20 wk after germination.

Transformation Experiments. Transgenic *A. thaliana* (Col) plants were constructed by the floral dip method (7). A background accession, Col, shows an early flowering phenotype due to low levels of *FLC* expression (8, 9). The *AhgFLC* coding sequences were amplified from two individuals, one from the study site (OMO) and the other from another population (INA, 34°90'N, 135°35'E). The *35S::AtFLC*, *35S::AhgFLC*^{OMO} and *35S::AhgFLC*^{INA} vectors were constructed by first introducing the corresponding sequences in the entry vector pCR/GW/Topo (Invitrogen). Then the coding regions were transferred with LR clonase (Invitrogen) to the destination vector pMDC32 to create the final constructs (10). The vectors were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation.

Seasonal *AhgFLC* Expression. Samples were collected at 1700 hours until November 13, 2006, and after that the sampling time was shifted to 1200 hours to avoid collecting samples after sunset. Analyses excluding data before the change of sampling time did not alter the results significantly. We therefore used the full data set in the analyses presented in the main text. For two of the six individuals, the level of *AhgFLC* expression was measured throughout the entire 2-y study period. One individual was replaced by a neighboring plant on November 6, 2006, due to the accidental death of an original plant, and then four individuals (including the one replaced) were replaced by the neighboring plants on February 19, 2007, to avoid excess damage by repeated sampling. On the latter replacement, we measured *AhgFLC* levels both for the original and replaced individuals on the same day, and found no significant differences between them on average. The 2-y study resulted in 576 measurements with three missing values. Samples collected from the natural population were immediately preserved in 150 μL of RNA stabilizing reagent (RNAlater; Ambion, Applied Biosystems) on ice. Following total RNA extraction, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for real-time quantitative PCR measurements (see Table S1 for primer information). The *AhgFLC* expression level was normalized against that of *ACTIN2* (11) by the comparative threshold cycle method (12) and was quantified relative to that of a 6-wk cold-treated standard sample. The standard plant was grown at 20 °C/15 °C from a seed for 8 wk before the cold treatments and then placed at 4 °C (see *Growth Experiment* for detailed growth conditions). We used the standard sample primarily because it allowed us to calibrate systematic errors that may have existed between different real-time PCR runs. In our preliminary experiments, the 6-wk cold induced the vernalization response in *A. halleri.*

Diurnal Expression. The *AhgFLC* expression was quantified for three plants every 3 h during 48 h on days close to either the vernal equinox (March 20–21, 2008) or autumnal equinox (September 22–23, 2008). Sampling and measurement procedures were the same as for the seasonal measurements.

Phenology. We tagged 160 naturally growing plants and recorded the onset dates of bolting, flowering, and inflorescence reversion at every field visit. Onset of bolting was defined as elongation of stems by longer than 3 mm. The gross morphology of meristems in the phenology observations corresponded with the *AhgAP1* expression. Expression of *AhgAP1* in the apical meristem was examined by RT-PCR for a subset of plants (Table S1). The *AhgAP1* sequence showed a 97.5% identity with *A. thaliana AP1* (13).

Time-Series Analyses. It has been reported that temperatures below 0 °C have lower effectiveness on vernalization when compared with temperatures ranging from 2 °C to 4 °C (14), and there have been chilling unit (CU) models that incorporate such an effect (15). We simply assumed that CU increases linearly with temperature decrease in the chilling accumulation and exponential decay models in this study, because the average daily temperature were below 0 °C for only 1 and 4 d for the first and second years, respectively.

Akaike's information criteria (AIC), as measures of the goodness of fit of the models, were also calculated (Table S2). For each data set given, three models were ranked according to AIC, with the one having the lowest AIC being the best. In the analysis using the air temperature record at the nearest meteorological station, a chilling accumulation model was selected according to AIC (Table S2), and the results of this model were presented in the main text. With the most likely L and T, the *AhgFLC* expression was regressed against the cumulative sum of CUs calculated based upon each model assumption, and adjusted R^2 was calculated to show how much of the variation in the *AhgFLC* expression was explained by the past temperature (Table S2). Average SEs of the *AhgFLC* expression in the transplant experiments from the model predictions were also calculated for the transplants to 20 °C/15 °C and 4 °C/4 °C conditions separately (Table S2).

The additional analyses using half-year data that cover either up-regulation (February to July 2007) or down-regulation periods (August 2007 to June 2008) had a shorter L (~20 d) for the former period than those (~60 d for the latter period (Table S2). An exponential decay model was selected for the up-regulation period, and chilling hour and chilling accumulation models were selected for the down-regulation period (Table S2).

Transplant Experiments. Plants with a single rosette were used in the transplant experiments. For each experiment, we selected six pairs of naturally growing plants. Two similar-sized adjacent plants were selected as a pair, and the two plants were designated to either of the two transplant conditions (see *Growth Experiment* for detailed growth conditions).

Semiquantitative RT-PCR. We determined the full-length cDNA sequences of *AhgFT* using the primers designed at UTR regions, and partial cDNA sequences for *AhgSOC1*, *AhgVIN3*, *AhgVRN2*, and *AhgLHP1* (Table S1). The *AhgFT*, *AhgSOC1*, *AhgVIN3*, *AhgVRN2*, and *AhgLHP1* sequences showed 96.8%, 98.9%, 95.0%, 96.8%, and 95.0% identities, respectively, with the corresponding *A. thaliana* homologs. RT-PCR was conducted using the selected cDNAs produced from samples collected during the first year of the field census.

Growth Experiments. All growth experiments in this study were conducted under the following conditions. Plants were grown in pots (75 mm in diameter) filled with culture soil [N, P, K: 0.4, 1.0, 0.4 g/kg soil (pH ~6.5)] and vermiculite mixture (1:3 in volume). In the 20 °C/15 °C (12/12-h day/night) condition, pots were placed in a growth cabinet (Koitotron HMN-S; Koito). The photosynthetically active radiation (PAR) was 83 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the red/far red ratio (660/730 nm) was 1.91. In the 4 °C (12/12 h day/night) condition, pots were placed in an incubator (model M-200F; TAITEC) with 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR and a 2.66 red/far red ratio.

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Fig. S1. The study was conducted in a natural population of perennial *Arabidopsis halleri* subsp. *gemmifera* in central Honshu, Japan (35°06′ N, 134°55′ E, altitude 190–230 m, shown by an arrow in *A*). (*B*) The census plot was established in the *A. halleri* population on an open site along a small stream that runs through secondary forest. (*C*) An *A. halleri* plant flowering in the study site.

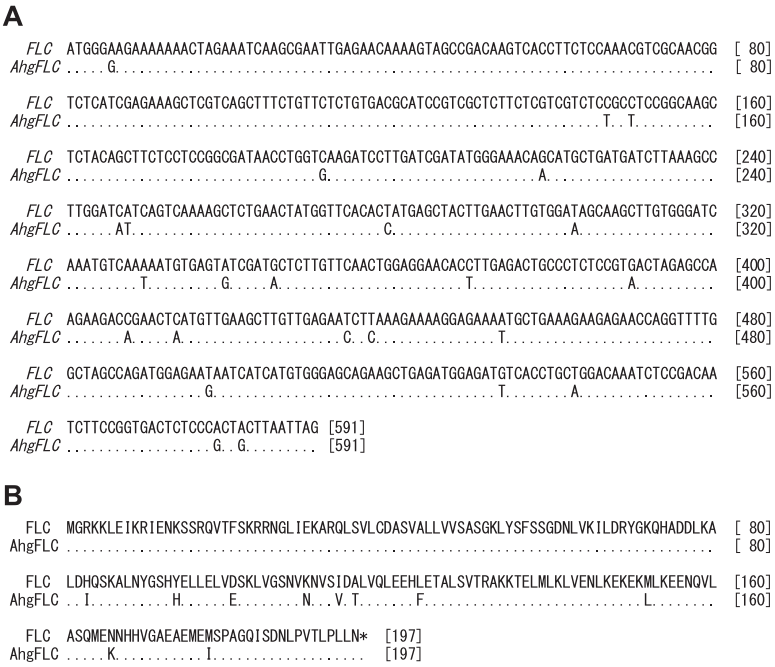

Fig. S2. *AhgFLC* full-length cDNA showed 95.9% and 94.9% identity in nucleotide (*A*) and amino acid (*B*) sequences, respectively, with *A. thaliana FLC*. Nucleotide and amino acid substitutions in *AhgFLC* are indicated by letters.

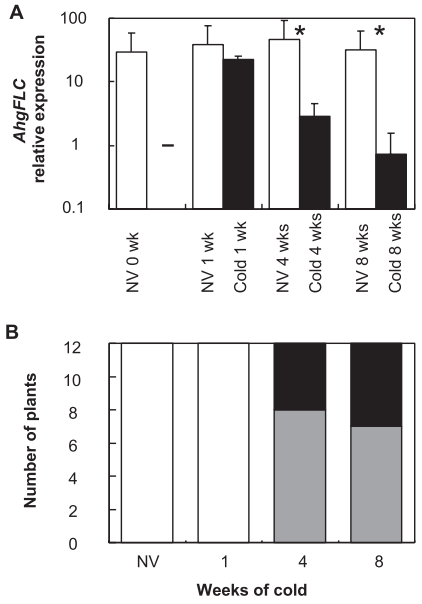

Fig. S3. Four-week or longer cold treatment suppressed *AhgFLC* expression (*A*) and promoted bolting and flowering of *A. halleri* (*B*). (*A*) *AhgFLC* transcription (mean and SD, $n = 6$) immediately after 1-, 4-, and 8-wk cold treatments (black bars) compared with that of the nonvernalized controls with the same timings (NV, white bars). Asterisks indicate a significant difference between cold treatment and NV in the *AhgFLC* expression ($P < 0.001$, ANOVA). (*B*) Gray and black areas represent numbers of bolted and flowered plants, respectively, during 12 wk from the start of the experimental treatment (20 wk after germination). Blank areas represent number of plants that remained as vegetative rosettes.

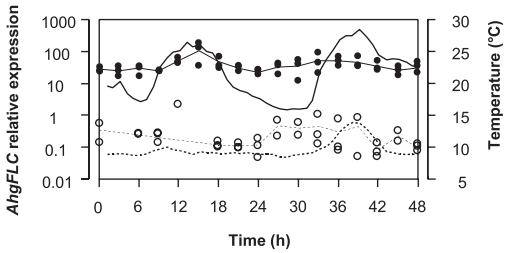

Fig. S4. The diurnal variation in the *AhgFLC* expression was much smaller than the seasonal variation. The *AhgFLC* expression (n = 3) measured by quantitative real-time PCR at 3-h intervals for 2 d close to either the vernal (open circles, March 20–21, 2008; the dotted thin line represents diurnal change on average) or autumnal (closed circles, September 22–23, 2008; the solid thin line represents diurnal change on average) equinox. Dotted and solid thick lines indicate air temperatures during the spring and autumn measurements, respectively.

Table S1. Primers used in this study

Genes	Experiments	Primers
AhgFLC	cDNA sequence	5′-GAGGATCAAATTAGGGCACAAAGC-3′
		5′-TAAGATATACAAACGCTCGCCCTTA-3′
	Real-time PCR	5′-CATCATGTGGGAGCAGAAGCT-3′
		5′-CGGAAGATTGTCGGAGATTTG-3′
	Transformation	5′-GCCATGGGGAGAAAAAAACTAGAAAT-3′
		5′-CTAATTAAGCAGCGGGAGAGTCACC-3′
	RT-PCR	5′-GAAACAACATGCTGATGATCTTAAA-3′
		5′-AAATCTCCATCTCAGCTTCTGCT-3′
AtFLC	Transformation	5′-GCCATGGGAAGAAAAAAACTAGAAAT-3′
		5′-CTAATTAAGTAGTGGGAGAGTCACC-3′
AhgAP1	RT-PCR	5′-TAGACAAGTGACATTCTCGA-3′
		5′-GAAGCAGCCAGGGTTGCAGT-3′
AhgFT	cDNA sequence	5′-AATCAACACAGAGAAACCACCTG-3′
		5′-TCATCACCGTTCGTTACTCGTAT-3′
	RT-PCR	5′-CAACCCTCACCTCCGAGAATATC-3′
		5′-CACCCTGGTGCATACACTGTTT-3′
AhgSOC1	cDNA sequence and RT-PCR	5′-TGAGGCATACTAAGGATCGAGTCAG-3′
		5′-GCGTCTCTACTTCAGAACTTGGGC-3′
AhgVIN3	cDNA sequence and RT-PCR	5′-ACTTGCTCGGATGCTGGAGAA-3′
		5′-GTAGAAGAAGACGGCTCCTCGG-3′
AhgVRN2	cDNA sequence and RT-PCR	5′-ACTGAAGTTAGGGAGGATTGTTCTT-3′
		5′-TTTCTGAAGAAGTAGGTGGCTTCTA-3′
AhgLHP1	cDNA sequence and RT-PCR	5′-TCGTCGTAAGAGAGTTCGAAAAG-3′
		5′-TGCACCGTTACTATTGTTGACTG-3′
AhgACTIN2	Real-time PCR	5′-TCCCTCAGCACATTCCAGCAGAT-3′
		5′-AACGATTCCTGGACCTGCCTCATC-3′
	RT-PCR	5′-AACGATTCCTGGACCTGCCTCATCATACTC-3′
		5′-AGAGATTCAGATGCCCAGAAGTCTTGTTCC-3′

Table S2. Comparison of three CU models using different sets of *AhgFLC* and temperature data

AhgFLC data	Temperature data	Models	AIC	L^a (95% CI)	T	R^2	Mean SE in the transplant experiments		
							20 °C/15 °C	4 °C/4 °C	All
September 2006 –August 2008 (2 y)	Meteorological station	**Chilling accumulation**	**858.6**	42 (39–45)	10.5 (9.7–11.1)	0.83	0.063	0.30	0.18
		Chilling hour	865.3	41 (38–42)	6.7 (6.7–7.5)	0.83	0.058	0.72	0.39
		Exponential decay	880.7	52 [0.044 (0.041–0.047)]	10.2 (9.3–10.9)	0.82	0.062	0.44	0.25
	Ground-surface at the site	Chilling accumulation	899.6	44 (42–45)	11.2 (10.4–11.8)	0.82	0.084	0.29	0.19
		Chilling hour	**896.0**	43 (42–51)	7.0 (5.5–7.0)	0.82	0.094	0.38	0.24
		Exponential decay	909.0	66 [0.035 (0.032–0.039)]	10.9 (10.1–11.5)	0.81	0.084	0.33	0.21
February 2007 –July 2007 (half-year up-regulation period)[b]	Meteorological station	Chilling accumulation	215.5	18 (8–23)	11.9 (8.8–14.0)	0.87	0.12	—	—
		Chilling hour	217.1	16 (9–23)	6.5 (5.1–8.7)	0.86	0.12	—	—
		Exponential decay	**210.8**	23 [0.099 (0.068–0.14)]	10.2 (8.3–12.8)	0.87	0.087	—	—
August 2007 –January 2008 (half-year downregulation period)[c]	Meteorological station	**Chilling accumulation**	**187.3**	58 (15–72)	11.8 (0.7–14.3)	0.78	—	0.47	—
		Chilling hour	**187.2**	64 (15–76)	9.0 (0.2–10.4)	0.78	—	0.34	—
		Exponential decay	193.2	105 [0.022 (0.017–0.052)]	11.3 (1.1–13.9)	0.77	—	0.59	—

For *AhgFLC*, data from either the entire 2-y period or half-year data that covers the up-regulation or down-regulation periods were used. For temperature records, either hourly air temperature at the nearest meteorological station or hourly ground-surface temperature at the study site was used. AIC are listed, and for each data set given, three models are ranked according to their AIC, the one with the lowest AIC being the best (shown by boldface letters, if the difference in AIC was smaller than two between the two best models, both models were selected). Maximum likelihood estimates of L (days) and T (°C) are listed with 95% confidence intervals (CI) in parentheses. Adjusted R^2 in the linear regressions of the *AhgFLC* expression against the cumulative sum of CUs with the most likely L and T are shown. Mean squared errors (SE) of the *AhgFLC* expression in the transplant experiments from the model predictions were also calculated for the transplants to 20 °C/15 °C and 4 °C/4 °C conditions separately, and a smaller SE represents a lesser difference between the observed and predicted value.

[a]For the exponential models, the results for the model parameter, α, is shown in brackets, and number of days that cover 90% of the whole effect is listed.

[b]Mean SE was calculated only for the transplant experiments to 20 °C/15 °C.

[c]Mean SE was calculated only for the transplant experiments to 4 °C/4 °C.