Clinal Variation in Flowering Time and Vernalisation Requirement across a 3000-M Altitudinal Range in Perennial Arabidopsis kamchatica ssp. Kamchatica and Annual Lowland Subspecies Kawasakiana

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Abstract

Clines in ecologically important traits across environmental gradients provide evidence of historical natural selection. Among the many adaptive traits that are relevant in natural habitats, flowering timing is a primary determinant of a plant’s lifetime fitness. In a laboratory experiment, we studied the divergence in flowering time and vernalisation requirement among 38 populations of two Arabidopsis subspecies: perennial A. kamchatica ssp. kamchatica and Annual Lowland subspecies, kawasakiana. Flowering time with vernalisation was not different and flowering time without vernalisation was slightly different between the subspecies; however, the altitude of the source populations was the dominant determinant of the variation in these traits for the species as a whole. The flowering time increased linearly and the effect of vernalisation to shorten the flowering time increased non-linearly with increasing altitude of the source population. Low-altitude populations of ssp. kamchatica from laboratory-collected seeds showed a stronger response to vernalisation than field-collected seeds, suggesting an effect of the maternal environment. By replicating the altitudinal gradient and explicitly accounting for the maternal effect, our results clearly suggest the existence of genetically based clinal patterns that provide signs of adaptive evolution. Early flowering is probably advantageous to completing reproduction before the hot summer at low altitudes. In ssp. kamchatica, which is perennial and can reproduce even if it does not flower in its first year, the strong vernalisation requirement may delay the first reproduction at high altitudes, where the growing season is short.

Keywords: Local adaptation; Altitudinal cline; Elevational cline; Altitudinal gradient; Elevational gradient; Maternal effect; Vernalisation; Wild relatives of Arabidopsis; Climatic gradient; Japanese Alps

Introduction

The adaptation of species to environmental gradients has been a central issue in ecology, ecomorphology, and evolutionary biology. Evidence of the action of natural selection over an environmental gradient can be provided by the existence of genetic clines in ecologically important traits along the gradient [1]. Among the many traits that are relevant to adaptation in natural environments, the timing of reproduction is a critical component of any organism’s life cycle, and in nature, it can be a primary determinant of an individual’s lifetime fitness [2]. Because the optimal timing of flowering may vary substantially along gradients related to latitude and altitude, which are both associated with the length of the growing season, the response of flowering to environmental cues is suspected to be a selection factor, leading to genetic divergence among populations. Life-history theory [3,4] assumes the existence of a trade-off between rapid reproductive development and maximum vegetative growth that results in increased future reproduction, and predicts that plants will flower earlier in environments with a shorter growing season. Indeed, many empirical studies have demonstrated the existence of latitudinal clines in which populations originating at higher latitudes flower earlier than those from lower latitudes when grown in a common environment [5-8]. On the other hand, the opposite pattern, in which populations from warmer locations flower earlier, have been shown for both latitudinal [9,10] and altitudinal [11] gradients. The adaptive significance of this opposite pattern can be explained by the relatively dry summer at warmer locations in these studies, because early flowering is often advantageous in habitats that experience drought in growing seasons [2,12-15].

The timing of flowering is often regulated by the existence of a winter season in strongly seasonal climates. Some plants require exposure to cold temperatures before they can flower after the winter; this well-known effect of the cold period is referred to as “vernalisation”. In this paper, we refer to the extent by which vernalisation shortens the flowering time compared with flowering in the absence of vernalisation as the “vernalisation requirement” of a species. An intra-specific genetic variation in the response to vernalisation has been reported in many species, including Arabidopsis thaliana [16,17], Arabidopsis lyrata [18], and the wild beet Beta vulgaris [19]. However, the degree of clinal variation in vernalisation requirement has been documented much less than flowering timing. In the annual plant A. thaliana, the vernalisation requirement is stronger in populations from lower latitudes, which respond to a shorter period of vernalisation than populations from higher latitudes [20]. These authors suggested that the low-latitude population might have evolved a higher sensitivity to vernalisation to respond to the milder winter signals. In contrast, populations of the wild beet, a perennial plant, have a stronger vernalisation requirement at higher latitudes, which is shown by the lower proportion of flowering

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plants in the absence of strong vernalisation [19]. These authors pointed out that the vernalisation requirement would regulate the age of first reproduction, and its optimal strength would be associated with the optimal age of first reproduction under a given length of growing season. Although it is hard to base a strong conclusion on limited studies, the discrepancy between these two reports may be associated with differences in the life forms of these plants. Although vernalisation should control the flowering season of annual plants, which have only one year in which to reproduce, it should control the year of first flowering as well as the flowering season of perennial plants.

Despite the usefulness of clinal variation to understanding the process of biological adaptation, two points should be carefully considered before the existence of clines is interpreted as evidence of natural selection. First, when clinal patterns are derived from a single altitudinal gradient, this can sometimes be caused by historical artefacts such as populations at low and high ends of a gradient that were founded by distinct ancestral lineages; this would generate the clinal pattern without requiring any adaptive evolution [11]. The same problem potentially exists for both altitudinal and latitudinal clinal patterns. These problems can be overcome by replication of the environmental gradients at different sites, since this increases the likelihood that at least some of the observed variation will be due to the consequence of natural selection rather than the result of founder effects. Second, when clinal patterns are assessed by growing field-collected seeds in a common garden or a laboratory, the effects of the maternal environment (hereafter, the "maternal effect") may contribute to any among-population differences that are observed [7,21,22]. Thus, proper assessment of genetic differences in any traits requires the use of seeds collected from mothers that experienced a common environment, thereby eliminating the maternal effect. However, only a fraction of the studies of clinal variation [7,10,11] in flowering time and vernalisation requirement have been fully or partly validated using this approach.

Here, we investigated the flowering traits of *Arabidopsis kamchatica* (Fisch. ex DC.) K. Shimizu & Kudoh, which is an allopolyploid plant derived from *A. lyrata* and *Arabidopsis halleri* [23]. Wild relatives of *A. thaliana*, which are ecologically diverse and genetically tractable, can serve as powerful models in evolutionary biology [24-26], and their use has become increasingly popular in this field [27-36]. *Arabidopsis kamchatica* harbours two ecologically diverse subspecies: *ssp. kamchatica* is a perennial plant with remarkably wide altitudinal distribution, ranging from 30 to nearly 3000 m, within a narrow latitudinal range (35.2°N to 36.8°N) in central Japan, whereas *ssp. kawasakiiana* is an endangered annual plant whose distribution is limited to lowland seaside and lakeside sites [37]. One of the main advantages of using altitude as the clinal parameter in ecological and evolutionary studies is that steep environmental gradients occur within short spatial distances, providing powerful natural experiments [38].

We investigated how flowering time and vernalisation requirement have diverged between these annual and perennial subspecies and across such a wide altitudinal gradient in *ssp. kamchatica*. To ensure replication of altitudinal gradients, we investigated 29 populations of *ssp. kamchatica* across five altitudinal gradients in different mountain systems of the Japanese Alps, as well as 9 populations of *ssp. kawasakiiana* from lowland populations. The Japanese Alps include six separate mountain chains or peaks with elevations around 3000 m, and therefore provide useful replication of altitudinal gradients that can be used in evolutionary studies. We used both field-collected and laboratory-generated seeds to explicitly account for the maternal effect. We grew plants in common laboratory environments with and without vernalisation. The divergence between the two subspecies can be examined by comparisons conducted at the same altitude. We predicted (i) that the annual subspecies would flower earlier, because rapid growth and early flowering would ensure reproduction of the annual plants but would decrease survival and reproductive output at later stages in the perennial plants [39-41]; and (ii) that the annual subspecies would have a weaker vernalisation requirement, because annual plants should be constrained to reproduce during their first year (i.e., their only year), irrespective of the strength and existence of a winter season, which are variable between years and depend on when they germinate. Also, the altitudinal divergence within the subspecies can also be examined by comparing populations of *ssp. kamchatica* across altitudinal gradients. We predicted (iii) that populations from higher altitudes would flower earlier than those from lower altitudes, because the length of the growing season and duration of suitable environmental conditions would be shorter at higher altitudes in central Japan, where the summer is generally moist and the snowfall is much greater at higher altitude; and (iv) that populations from higher altitudes would have a stronger vernalisation requirement, because the optimal age of starting reproduction would be older for the perennial subspecies owing to the shorter growing season and the need to allocate resources to early survival and growth in a difficult environment rather than to reproduction.

**Materials and Methods**

**Plant materials and climatic census**

*Arabidopsis kamchatica* is self-compatible herb and capable of autogamy [37]. *Ssp. kawasakiiana* is winter annual that germinates in autumn or winter and flowers in spring. *Ssp. kamchatica* is perennial that flowers in spring at low altitude and in summer at high altitude. Newly established seedlings of *ssp. kamchatica* were observed both in autumn (from September to November) and in spring and summer (from May to August), suggesting that the seeds germinate both before and after winter in this subspecies (Y. Onda & T. Kenta, unpublished data). The variation in germination timing along altitude has not been known.

We explored natural populations of *ssp. kamchatica* by exploiting information on specimens that were available in museums and herbariums (Table 1). Using this information, we chose 29 populations that covered a wide range of altitudes (from 30 to 2949 m) around three mountain chains and two single-peak mountains in the Japanese Alps (Figure 1). Seeds of *ssp. kamchatica* were collected from these populations in both 2008 and 2009. We obtained all required permits for collection of seeds at these locations. Seeds of *ssp. kawasakiiana* from the nine populations were kindly provided by researchers at Kyoto University (Table 1). All seeds of the both subspecies were preserved separately for each maternal plant. Part of those seeds were grown in 2009 in the same laboratory under growing conditions similar to those used in the flowering experiment described below, and the flowers were bagged to produce self-pollinated seeds. Seeds of each maternal plant were again preserved separately so that each lineage was descended from a single maternal plant from the field. Hereafter, plants from the field-collected seeds and those from the lab-derived seeds are referred to as the first and second laboratory generations, respectively. Maternal plants of the first laboratory generation experienced a variable field environment, whereas those from the second generation experienced a common laboratory environment. If flowering timing differed between...
<table>
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<tr>
<th>No.</th>
<th>Population</th>
<th>Subspecies</th>
<th>Latitude (°N)</th>
<th>Longitude (°E)</th>
<th>Altitude (m)</th>
<th>Specimen No.¹</th>
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</table>

¹Specimen number from museum and herbarium collections that provided information on the locations of the natural populations from which we collected seeds. If we did not use such information to find a population, our own specimen number (Y. Onda) is shown. Abbreviations: TOYA, Toyama Science Museum; KYO, Kyoto University Museum; SHIN, Shinshu University Herbarium; TNS, National Museum of Nature and Science; MAK, Tokyo Metropolitan University; ncu, seeds were not collected by us, and were instead provided by Dr. J. Sugisaka and Mr. M. Yamaguchi of Kyoto University.

Table 1: Location of the 38 A. kamchatica populations from which seeds were collected. The number of each population corresponds to the number in Figure 1.
these generations, the maternal effect could be involved.

In each population of ssp. *kamchatlica*, we monitored soil temperatures at a depth of 5 cm using an iButton DS1921G temperature datalogger (Maxim, Sunnyvale, CA, USA) at 2-h intervals for 40 - 90 days from July to September of 2009. Because census duration differed between populations, we only analysed the maximum temperature in the duration. After eliminating datasets from dataloggers that broke or were moved, we obtained data for 22 of the populations.

**Flowering experiment**

For ssp. *kamchatlica*, we sowed a total of 90 seeds obtained from 1 to 7 lineages per population from each of 14 and 18 populations selected to cover the full range of altitudes of the source populations from the first and second laboratory generations, respectively; 12 of these populations were the same in both generations. For ssp. *kawasakiana*, we sowed a total of 90 seeds obtained from 1 to 7 lineages from each of the 9 populations in the second laboratory generation (Table 2). Seeds of each generation of each population were uniformly divided into 3 treatments with different growing conditions: (1) a 22°C day temperature (22DT), (2) a 22°C day temperature with a constant 5°C vernalisation treatment for 4 weeks before return to the 22°C day temperature (22/5/22DT), and (3) a 15°C day temperature with a constant 5°C vernalisation treatment for 4 weeks before return to the 15°C day temperature (15/5/15DT). In all treatments, the light period was 20 h per day, with 4 h of night and a night temperature of 16°C.

For the 22/5/22DT and 22DT treatments, fluorescent lamps suitable for plant growth (FL40SBR-A and FL20SBR-A; NEC, Tokyo, Japan) were used to provide photosynthetically active radiation (PAR) of approximately 120 µmol/m²/s in average. The 15/5/15DT treatment was conducted in another growth chamber with a uniform mixture of FL40SBR-A fluorescent lamps, FLR40S-EX-N fluorescent lamps (from Toshiba, Tokyo, and Panasonic, Kadoma, Japan), and FL40SD fluorescent lamps (NEC, Tokyo). PAR was approximately 60 µmol/m²/s in average. Plant trays were moved and rotated twice each week so that they experienced more uniform light and other micro-environmental conditions.

Each seed was sown on a 3-cm cube of rockwool (Nittobo, Tokyo) on a depth of approximately 1 cm of vermiculite (Nittai, Osaka). Plants were watered with the mixture of 1/2000 Hyponex 6-10-5 (containing 1.0 mM ammonium-N, 0.4 mM nitrate-N, 0.7 mM other N, 0.5 mM phosphoric acid, 0.6 mM K, and micronutrients; Hyponex Japan, Osaka) and 0.75 mM magnesium sulphate twice a week. Every time of watering, the fertiliser solution reached a height of approximately 3 cm from the bottom of the trays. The solution included 5 g/L acephate, which was provided once every 2 weeks to control flies. When plants
Table 2: Numbers of lineages and of plants per lineage (in parentheses) used in this study. Population numbers are the same ones shown in Table 1.

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<th>No.</th>
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<th>Flowering plants</th>
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<td>2nd gen.²</td>
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<td>kam.</td>
<td>7 (4-18)</td>
<td>7 (12-13)</td>
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<td>kam.</td>
<td>3 (30-30)</td>
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<td>15</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>16</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>17</td>
<td>kam.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>18</td>
<td>kam.</td>
<td>5 (7-30)</td>
<td>5 (18-18)</td>
</tr>
<tr>
<td>19</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>kam.</td>
<td>-</td>
<td>2 (30-60)</td>
</tr>
<tr>
<td>22</td>
<td>kam.</td>
<td>-</td>
<td>1 (90)</td>
</tr>
<tr>
<td>23</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>kam.</td>
<td>4 (14-28)</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>26</td>
<td>kam.</td>
<td>5 (9-27)</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>27</td>
<td>kam.</td>
<td>-</td>
<td>2 (30-60)</td>
</tr>
<tr>
<td>28</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>kam.</td>
<td>3 (30-30)</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>31</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>32</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>33</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>34</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>35</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>36</td>
<td>kaw.</td>
<td>-</td>
<td>2 (30-60)</td>
</tr>
<tr>
<td>37</td>
<td>kaw.</td>
<td>-</td>
<td>3 (30-30)</td>
</tr>
<tr>
<td>38</td>
<td>kaw.</td>
<td>-</td>
<td>4 (15-30)</td>
</tr>
</tbody>
</table>

¹Subspecies kamchatica (kam) or ssp. kawasakiana (kaw). 2Growing conditions: 22DT, 22°C day temperature; 22/5/22DT, 22°C day temperature with a 5°C vernalisation treatment (4 weeks) followed by a return to the 22°C day temperature; 15/5/15DT, 15°C day temperature with a 5°C vernalisation treatment (4 weeks) followed by a return to the 15°C day temperature. See the Methods section for details. 3 Laboratory generations: 1st, plants from seeds collected in the field; 2nd, plants from seeds of laboratory-grown plants.

Table 2: Numbers of lineages and of plants per lineage (in parentheses) used in this study. Population numbers are the same ones shown in Table 1.
reached at least the six-leaf stage (~1 month after germination), plants in the 22/5/22DT and 15/5/15DT treatments were vernalised at 5°C for 4 weeks, followed by transfer to 125-cm³ cubical pots filled with vermiculite (Nittai, Osaka) under the same temperature conditions as used before vernalisation. Plants in the 20DT treatment were directly transferred into pots at the same growth stage as the other plants, but with no vernalisation treatment. We observed plant germination and flowering weekly and used the number of days from germination to flowering as the flowering time.

Statistical analyses

The relationship between soil temperature and altitude was fitted using a non-linear model, the nls() function, provided by version 2.13.0 of the R statistical software [42]:

$$MT = \beta_0 + \beta_1A + \beta_2A^2$$

where $MT$ is the summer maximum of the daily minimum, mean, and maximum soil temperatures (in separate regressions); $I$ is the temperature intercept; $A$ is the altitude; and $\beta_i$ (for $i = 0$ to 2) is the regression coefficient for each explanatory variable. Variable selection was conducted by calculating the value of Akaike’s Information Criterion (AIC) for all possible combinations of variables. The model with the lowest AIC was selected as the best model.

Flowering time in the 22DT and 22/5/22DT treatments was analysed simultaneously because these treatments were identical apart from the presence of vernalisation in the latter treatment. The effect of vernalisation was explicitly examined. We then analysed the 15/5/15DT treatment separately because it was conducted in a different growth cabinet, so the growth conditions may have been different from those in the first growth cabinet. Flowering time in the 22DT and 22/5/22DT treatments was fitted to altitude, vernalisation, laboratory generation, and subspecies by using a non-linear mixed-effects model, the lme() function, provided by the R software [42]:

$$F = \beta_0I + \beta_1A + \beta_2A^2 + \beta_3 + \beta_4G + \beta_5S + \text{interactions}$$

where $F$ is the number of days to flowering, $I$ is the random effect on the intercept of population and lineage within the population, $A$ is altitude, $V$ is vernalisation (0 without, 1 with vernalisation), $G$ is the laboratory generation (0 for first, 1 for second laboratory generation), $S$ is the subspecies (0 for kamchatica, 1 for kawasakiana), and $\beta_i$ (for $i = 0$ to 5) is the coefficient of each explanatory variable. In addition,

<table>
<thead>
<tr>
<th>AIC of each model</th>
<th>Summer maximum value of daily temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum temperature</td>
<td>Mean temperature</td>
</tr>
<tr>
<td>Null model</td>
<td>135.7</td>
</tr>
<tr>
<td>Linear regression as a function of altitude</td>
<td>76.3</td>
</tr>
<tr>
<td>Quadratic regression as a function of altitude</td>
<td>74.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Estimates in the best-fit model</th>
<th>Summer maximum value of daily temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>2.78E+01 ***</td>
</tr>
<tr>
<td>Altitude</td>
<td>-6.92E-03 ***</td>
</tr>
<tr>
<td>Altitude²</td>
<td>6.02E-07 +</td>
</tr>
</tbody>
</table>

Table 3: AIC values and estimates of parameters for the models used to fit soil (5-cm depth) temperature to altitude.
we included all possible interactions between variables in the model except those between A and A² (to avoid autocorrelation), between A and S and between A² and S (because ssp. kawasakiana was limited to low-altitude sites), and between G and S (because ssp. kawasakiana was composed entirely of the second laboratory generation). Variable selection was conducted by calculating AIC for all the remaining combinations of main and interactive effects of variables, and the model with the lowest AIC was selected as the best model.

Flowering time in the 15/5/15DT treatment was fitted to the same model as that in the 22DT and 22/5/22DT treatments, with all the same variables except for V (vernalisation), because there was no corresponding control treatment without vernalisation.

Results

Three measures of the summer maximum soil temperature (minimum, mean, and maximum) were quadratically fitted to altitude (Table 3). The AICs of the quadratic models were slightly (1.4 to 1.8) lower than those of the linear model and greatly (4.9 to 60.8) lower than those of the null model. The estimated coefficient of the square of the altitude (A²) was smallest for the daily minimum temperature, followed by those of the daily mean and maximum temperatures. The quadratic regression lines with smaller coefficients of A² were closer to linear (Figure 2). The three measures of the summer maximum soil temperature decreased with increasing altitude, but the daily maximum temperature increased slightly above an altitude of 2000 m.

The best model indicates that flowering time was affected by altitude (A) and interactions between vernalisation and each of altitude (V × A), square altitude (V × A²), laboratory generation (V × G), subspecies (V × S) and both the altitude and generation (V × A × G) (Table 4). All of these variables were highly significant, and AIC was improved to a large degree by the inclusion of each of those variables (Table 4). This means that in the absence of vernalisation (Figure 3, left), flowering time was fitted linearly to altitude, whereas in the vernalised treatment (Figure 3, middle), it was fitted quadratically to altitude with the effects of laboratory generation and subspecies. The V × A × G interaction indicates that the effect of the laboratory generation changes with altitude only when the plants have been vernalised. In the 15/5/15DT treatment, flowering time was fitted quadratically to altitude without the effects of laboratory generation and subspecies (Table 5; Figure 3, right). The effects of altitude was highly significant (P < 0.001), and inclusion of the variable largely improved AIC. The effects of A² was significant (P = 0.018), and inclusion of the variable considerably improved AIC.

Discussion

The divergence between subspecies and the maternal effect

Based on the best model, flowering time did not differ between the two subspecies when they were not vernalised, and populations of both subspecies could therefore be regressed using the same linear equation...
as a function of altitude (Figure 3, left). This suggests that the flowering time has not diverged between the two subspecies, and that altitude is the main determinant of flowering time for the species as a whole when the plants have not been vernalised. This contradicts our prediction that the annual subspecies would flower earlier than the perennial subspecies under the same growing conditions; the reason is discussed later in this section, along with the effect of vernalisation.

When the plants were vernalised, the model that the second laboratory population of sspp. kamchatica shortened flowering time at the lower end of the range in source altitude was selected (Figure 3 middle, yellow line), and $V \times G$ and $V \times A \times G$ interactions were highly significant (Table 4). However, because our dataset lacked low-altitude populations of sspp. kamchatica in the second laboratory generation, further study will be required to confirm the difference between the laboratory generations. Important point is, the effect of altitude and vernalisation found by this study is not violated by the maternal effect because those effects were detected independently from laboratory generations.

In contrast to the second laboratory generation of sspp. kamchatica, which exhibited shorter flowering time after vernalisation at the low altitude, the flowering time of the same generation of sspp. kawasakiana was not greatly changed by the vernalisation. The difference between subspecies in an environment with vernalisation (i.e., the $V \times S$ interaction; Table 4) was statistically significant. This result is consistent with our prediction that the perennial subspecies would have a stronger vernalisation requirement. However, we again lacked data for the low-altitude sspp. kamchatica populations in the second laboratory generation, which would have permitted a direct comparison between the subspecies. The estimated difference between subspecies was also small (14 days), and sspp. kawasakiana did not show a large deviation from the overall relationship between flowering time and the altitude of the source population that the second laboratory generation of sspp. kamchatica showed. This overall pattern suggests that the flowering time of the whole species (both subspecies combined) after vernalisation is determined more strongly by the altitude of the source population than by the difference between the subspecies.

We recently found that the annual field survival rate of the perennial sspp. kamchatica was much lower at lower altitudes, mainly owing to higher summer mortality (Y. Onda & T. Kenta, unpublished data). This result suggests that the subspecies has a life history that resembles that of an annual plant at low altitudes; however, because sspp. kawasakiana is a typical annual plant, its annual survival rate is zero, which is still lower than that of the low-altitude populations of sspp. kamchatica. The absence of divergence in flowering time between the subspecies when the plants are not vernalised and the relatively weak subspecies divergence in vernalisation requirement are consistent with the small difference in life history between the subspecies at low altitudes. Further studies will be required to understand the extent of the ecological divergence between these subspecies.

Altitudinal cline in sspp. Kamchatica

Flowering time increased linearly with increasing altitude of the source population when the plants were not vernalised (Table 4; Figure 3, left). The effect of vernalisation also differed as a function of altitude, as indicated by the significant $V \times A$ and $V \times A^2$ interactions (Table 4), and the flowering time with the vernalisation treatment increased quadratically with increasing altitude of the source population (Figure 3, middle). The magnitude of the dispersion the observed data from the regression line decreased greatly when the plants had been vernalised, suggesting that genetic variation that is not determined by the altitude of the source population is exposed in the absence of vernalisation, although the reason for this is unclear.

The difference in regressed flowering time between the 22DT and 22/5/22DT treatments increased quadratically with increasing altitude of the source population (Figure 3, left and middle) owing to the negative coefficient of the $V \times A$ interaction, suggesting that plants from high altitudes have a stronger vernalisation requirement for flowering. Although the flowering time in the 15/5/15DT treatment was more variable than that in the 22/5/22DT treatment, possibly owing to the difference in growth chambers between these treatments, the single regression curve for the 15/5/15DT treatment was intermediate between the two regression curves for the two laboratory generations in the 22/5/22DT treatment (Figure 3, middle and right). Because the relationship between flowering time after vernalisation and the altitude of the source population was reproduced in two treatments under different growing conditions, this strongly supports our prediction that plants from higher altitudes would have a stronger vernalisation requirement for flowering. Because plants experienced the same environment in each treatment, and because we explicitly incorporated the maternal effect, these differences in flowering traits along an altitudinal gradient can be attributed to genetic differences. Also, because we replicated the altitudinal gradients by selecting gradients from five different locations, we can exclude the possibility of historical artefacts [11], i.e. the effects of a genetic structure associated with colonisation history. Thus, our results strongly suggest that the observed clinal variation is a sign of evolutionary adaptation of A. kamchatica to certain environments that are generally associated with altitude per se.

Interestingly, the finding that populations from a higher altitude flowered earlier contradicts our prediction and the results of many previous studies [5-8]. The adaptive significance of this result can be easily explained if lower locations are at greater risk of drought, because early flowering has been repeatedly found to be advantageous in habitats that experience drought during the growing season [2,12-15]. Although more snowfall and a longer period of snow cover could provide snowmelt water for longer periods at higher altitudes, the summer precipitation is generally high in Japan and we cannot confirm whether drought is more severe at lower altitudes at our study sites.

Another factor that may favour early flowering at low altitude is the hot summer temperature. At our study sites, the summer soil temperature tended to be higher at lower altitude, with a maximum that sometimes exceeds 35 or 40°C (Figure 2). In A. thaliana, exposure to a daily maximum air temperature of 40°C decreases seed set to less
than half of that of control plants, and to only a few per cent of the control level in particularly sensitive accessions, partly on account of the failure of pollen production [43]. In addition, summer mortality of ssp. kamchatka in the field has been found to increase at lower altitudes (Y. Onda & T. Kenta, unpublished data). Because central Japan is located near the southern limit of the distribution of this subspecies, which ranges from ca. 33°N to 65°N across East Asia, Russia, and North America [44], the hot summer temperature probably represents a major environmental challenge for it at low altitudes in central Japan. This can explain the advantage of early flowering, which would let the plants complete reproduction before the severe summer temperatures begin. A similar clinal pattern, in which plants from populations at warmer locations flower earlier, has been found in A. thaliana [10,11] and A. lyrata [9], in contrast to many other plants, which show the opposite pattern. The addition of our results to the literature raises the question of whether avoiding hot summer temperatures are a common and significant life history strategy in this genus.

Our prediction that the vernalisation requirement of ssp. kamchatka would be stronger at higher altitudes was supported by our results. Our study sites at high altitude are covered with snow for as long as 9 to 10 months, whereas some sites at low altitude have snow cover for as short as 1 to 2 months, and the length of the plant’s growing season therefore differs drastically as a function of altitude. In the field, newly established seedlings of ssp. kamchatka were observed both in autumn and in winter, suggesting that the seeds germinate both before and after winter (Y. Onda & T. Kenta, unpublished data). The growing season at high altitude is probably not sufficiently long to let plants that germinate after winter complete their reproduction, and a strong vernalisation requirement would prevent those plants from flowering during their first year. In addition, the optimal age for starting reproduction in perennial plants should be associated with the length of the growing season, and the vernalisation requirement may exert some control on this age. These interpretations are congruent with a similar clinal pattern in which the vernalisation requirement increased with the latitude of the source population in the perennial wild beet [19] and showed the opposite pattern in the annual A. thaliana [20]. Further studies will be required to evaluate whether stronger vernalisation at colder locations would be a common pattern in perennial plants.

Another possible explanation for the clinal variation in the vernalisation requirement is the genetic association between the flowering time without vernalisation and the vernalisation requirement. The flowering pathway of A. thaliana is one of the best-characterized genetic networks in plants, and genes involved in the autonomous, photoperiod, vernalisation, and gibberellin pathways are known to regulate, in an integrated manner, the expression of downstream genes that trigger flowering [45]. The flowering pathway is expected to be strongly conserved in related species. For example, sequence differences in FRI are responsible for variation in the vernalisation requirement in both A. thaliana [46] and A. lyrata [18]. In A. thaliana, accessions that flower later, when not vernalised, tend to have stronger vernalisation sensitivity, and a genetic correlation between these two phenotypes is suspected [20]. If the same genetic regulation is conserved in A. kamchatka, selections that favour later flowering at higher altitude might lead to the evolution of a stronger vernalisation requirement, or vice versa.

The flowering time of the vernalised plants showed a convex-upward non-linear curve (Figure 3, middle and right). This pattern may be associated with non-linearity in some environmental conditions for the source populations along the altitudinal gradient. For example, daily maximum and mean temperatures showed a stronger non-linear trend as a function of altitude than the daily minimum temperature showed (Figure 2). Non-linearity in the summer maximum temperature can be explained by the fact that the study sites at the highest altitudes are on mountain ridges that receive strong direct sunlight for most of the day, whereas study sites at middle altitudes are on slopes that are shaded by the ridges and trees for a longer portion of the day. The snow-cover season is also probably longer at mid-altitude sites (snowbed slopes) than at the highest altitudes (fellfield ridges). The distribution of ssp. kamchatka is sporadic, and populations on ridges and those on slopes are usually not continuous, suggesting that these populations may have experienced different suites of environmental conditions for a long time.

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