Association between RsFT, RsFLC and RsCOL5 (A&B) expression and flowering regulation in Japanese wild radish

Qingxiang Han1,*, Shota Sakaguchi2, Tomomi Wakabayashi2, Hiroaki Setoguchi2

1College of Life Sciences, Zaozhuang University, Zaozhuang City, Shandong Province, 277160, China, 2Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, 606-8501, Japan

*Corresponding author e-mail address: qingxianghan@163.com

Abstract

Flowering is an important step in the life cycle of plants and indicates adaptability to external climatic cues such as temperature and photoperiod. We investigated the expression patterns of core genes related to flowering-time regulation in Japanese wild radish (Raphanus sativus var. raphanistroides) with different vernalization requirements (obligate and facultative) and further identified climatic cues that may act as natural selective forces. Specifically, we analysed flowering-time variation under different cold and photoperiod treatments in Japanese wild radish collected from the Hokkaido (northern lineage) and Okinawa (southern lineage) islands, which experience contrasting climatic cues. The cultivation experiment verified the obligate and facultative vernalization requirements of the northern and southern wild radish accessions, respectively. The expression of major genes involved in flowering time indicated that RsFLC and RsCOL5 (A&B) may interact to regulate flowering time. Notably, floral initiation in the northern lineage was strongly correlated with RsFLC expression, whereas flowering in the southern lineage was correlated with induction of RsCOL5-A expression, despite high RsFLC transcript levels. These results suggested that the northern accessions are more sensitive to prolonged cold exposure, whereas the southern accessions are more sensitive to photoperiod. These different mechanisms ultimately confer an optimal flowering time in natural populations in response to locally contrasting climatic cues. This study provides new insights into the variant mechanisms underlying floral pathways in Japanese wild radish from different geographic locations.

Keywords: Flowering time; natural selection; photoperiod; vernalization; wild radish.

Introduction

Flowering is the developmental turning point from the vegetative to reproductive phase. The induction of flowering is the most important stage regarding reproductive strategy and the allocation of limited resources (Komeda 2004). Variation in flowering time is a key feature of the life histories of flowering plants and determines crucial aspects of plant reproductive ecology. As such, there has been intense interest in determining the genetic architecture of this trait (Fitter and Fitter 2002; Schlenker and Roberts 2009; Li et al. 2010; Raman et al. 2016; Xiao et al. 2019). In Arabidopsis thaliana, for example, the precise timing of flowering transition is regulated by a complex hierarchical signalling network that integrates many environmental and endogenous stimuli (Huijser and Schmid 2011; Weber and Burow 2018).
Various factors, including photoperiod, temperature, plant age and gibberellic acid (GA) content, converge to regulate FLOWERING LOCUS T (FT) expression in flowering plants (Fornara et al. 2010; Cho et al. 2017). Among multiple seasonal cues, temperature plays an essential regulatory role, particularly in plants that require an extended cold period to initiate flowering, known as vernalization (Michaels and Amasino 2000). A key component in the vernalization regulatory network in Arabidopsis is FLOWERING LOCUS C (FLC), a MADS-box transcription factor that quantitatively inhibits floral transition by encoding a FT transcription repressor (Michaels et al. 2003; Deng et al. 2011). FLC plays a critical role in monitoring seasonal temperature trends under fluctuating natural environments (Satake et al. 2013; Zheng et al. 2018). Vernalization stably represses FLC expression in response to prolonged cold exposure, and thus accelerates flowering, whereas FRIGIDA (FRI) promotes high FLC expression and thereby prevents flowering (Michaels and Amasino 2001; Searle et al. 2006). Allelic variations in FRI and FLC account for much of the natural variation in Arabidopsis flowering time (Shindo et al. 2005). For example, the winter-annual ecotypes contain dominant FRI and FLC alleles and require vernalization for rapid flowering (Michaels and Amasino 2000), whereas many summer-annual and rapid-cycling ecotypes flower quickly without vernalization via disruption of the FLC regulatory sequences (Michaels et al. 2003) and absence of an active FRI allele resulting in low FLC expression (Johanson et al. 2000).

In addition to temperature, photoperiod (day length) is a major environmental factor that affects the timing of floral transition. Under long-day light condition in Arabidopsis, molecular genetics has revealed that CONSTANS (CO), a transcription factor with two B-box-type zinc fingers, plays a central role in the photoperiod pathway, and its expression is controlled by both the circadian clock and light signals (Suárez-López et al. 2001; Böhlenius et al. 2006). And CO functions as a transcription factor that promotes flowering by inducing the expression of the florigen gene (FT) (Kardailsky et al. 1999; Corbesier et al. 2007; Kimmonth-Schultz et al. 2016). CO is one of 16 CO-LIKE (COL) genes that have been identified in the Arabidopsis genome (Robson et al. 2001). However, it has been shown that CO function may not be conserved in other COL proteins which contain B-boxes that are closely related to those of CO. For instance, changes in the expression levels of COL1 and COL2 have little effect on flowering time, whereas overexpression of COL1 can impact circadian rhythm (Ledger et al. 2001), fruit ripening and stress responses (Chen et al. 2012). COL3 is a positive regulator of red light signalling and root growth (Datta et al. 2006), and overexpression of COL9 functions as a floral repressor (Cheng and Wang 2005). In contrast, COL5, a diurnal and circadian-regulated member of the COL family of proteins, induces flowering time by increasing FT expression in short-day grown Arabidopsis (Hassidim et al. 2009).

The radish (Raphanus genus) is one of the most important and popular vegetable crops in the Brassicaceae family. It is closely related to Arabidopsis and shows considerable genetic homology (Donovan et al. 2006). Distinct from cultivated radish, the winter-annual wild radish (R. sativus var. raphanistrum) is a principal vernalization-responsive plant (Sung and Amasino 2005) that grows spontaneously and widely along coastlines of Japan. There is close relationship between wild radish and cultivars (Yamagishi 2004; Yamane et al. 2009; Wang et al. 2015), indicating that increased understanding of the flowering characteristics of wild radish should contribute to the manipulation of crop environments to promote synchronous and effective flowering in cultivated production. Wild radish plants in northern and southern Japan exhibit obligate and facultative vernalization, respectively (Han et al. 2016). Specifically, northern populations require obligate vernalization for flowering, whereas southern populations display facultative vernalization, i.e., can flower without cold exposure. However, vernalization hastens the flowering of these ecotypes, thus offering an excellent system for genetic studies of flowering-time variations. Various studies have demonstrated the gene network mechanism that controls flowering time in winter-annual and summer-annual ecotypes of A. thaliana (Johanson et al. 2000; Michaels et al. 2003; Michaels et al. 2005; Vidigal et al. 2016; Lee et al. 2019). However, the genetic mechanisms underlying flowering-time variation in plants with obligate and facultative vernalization requirements, and the possible floral pathways and molecular mechanisms related to adaptive variations remain less clear.

In the current study, we investigated the expression patterns of genes that control flowering time in Japanese wild radish with different vernalization requirements (obligate and facultative) and identified the climatic cues that may act as natural selective forces shaping genetic variation in floral initiation. Specifically, we analysed of variations in flowering time in response to different cold and day-length treatments in accessions collected from the Hokkaido (northern lineage) and Okinawa (southern lineage) islands with contrasting climatic conditions. Understanding the integration of vernalization and photoperiod is important for clarifying how plants alter flowering time in response to various environmental signals. This work should improve our knowledge of how plants regulate flowering in response to multiple environmental cues.

Materials and Methods

Measurement of flowering time

To document flowering time in wild radish, ripe seeds were collected from Hokkaido Island and Okinawa Island (Fig. 1) as representative lineages. The genetic structure and north-south lineage of the two populations were proven by molecular genetics, as described in our previous paper (Han et al. 2016). To avoid the influence of genetic variation among individuals on gene expression, we collected seed samples from different closely spaced plants and then pooled eight individuals per site as a replicate. The effects of cold exposure (with and without) and day length (long- and short-day) on flowering induction were evaluated by using a 2 × 2 factorial experiment. The seeds were sown in Petri dishes containing filter paper soaked in tap water under darkness conditions at 21 °C for 3 days. The seedlings were then transplanted into jiffy pots and placed within a growth chamber (21 °C, 10-h light/14-h dark) for vegetative development for 1 week.

In the vernalized group, 1-week-old seedlings received cold treatment at 5 °C (10-h light/14-h dark) for 3 weeks. After vernalization, the plants were transplanted into plastic pots (diameter 9 cm × height 20 cm), then moved to an air-conditioned greenhouse (21 °C) in Kyoto, Japan (35°01’N/135°46’E) and subjected to natural short- or long-day light conditions. Under each experimental treatment of day length, 1-week-old seedlings without cold exposure were simultaneously grown in the same greenhouse as vernalized group. The short-day light treatment was from October to January of the following year, and long-day light treatment was from April to July. To reduce the effects of position, the pots were moved randomly every week. Flowering time was measured as the number of days from greenhouse planting...
until the appearance of the first flower. Based on our observations, the growing period for most samples ended ~100 days after greenhouse planting. Hence, the experiment was terminated at 120 days.

The flowering data were imported into SPSS v26.0. To compare the different groups and treatments, one-way analysis of variance (ANOVA) and LSD tests (least significant difference tests) were performed. The level of significance was set to 5%. Additionally, Student’s t-test was used to determine significant differences between the long- and short-day length treatments, as well as between the vernalized and non-vernalized treatments. We also investigated the major climatic factors that may act as natural selective forces shaping variation in flowering time, including temperature and photoperiod. Climatic data from 1981 to 2010 were obtained from the Japan Meteorological Agency (JMA) located on Hokkaido and Okinawa islands, according to the World Meteorological Organization (WMO) Technical Regulations. Additionally, the mean day length per month in Hokkaido, Okinawa and Kyoto (location of cultivation experiment) was collected from weather station (https://weather-stats.com/japan). These data are presented in Supporting Information—Table S1 and Fig. S3.

Flowering-time gene expression analyses
To better understand the roles of cold exposure and photoperiod in determining flowering time in wild radish, we investigated the expression levels of major flowering-time genes, including RsFLC, RsCOL5 (A&B) and RsFT. The functions of the studied RsFLC, RsCOL5 (A&B) and RsFT genes are conserved between Arabidopsis and wild radish (Yi et al. 2014; Kitashiba and Yokoi 2017; Hu et al. 2018), and play key roles in the vernalization pathway, photoperiod pathway and flowering pathway integration, respectively (Putterill et al. 2004; Nie et al. 2016). We aligned four homologs of the COL5 gene (RSG21446.t1, RSG35888.t1, RSG44241.t1 and RSG42288.t1) in wild radish to AT5G57660.1 in Arabidopsis and the identity were 79.47, 79.42, 15.64 and 14.98 %, respectively. We eventually selected two genes with higher alignment, RSG35888.t1 (RsCOL5-A) and RSG21446.t1 (RsCOL5-B), as indicators of CO-LIKE 5. Supporting Information—Table S2 showed the corresponding homologs of FLC, COL5 (A&B) and FT in R. sativus (https://www.ncbi.nlm.nih.gov/assembly/GCA_001047155.1) and A. thaliana (http://www.arabidopsis.org/).

The seeds collected from Hokkaido and Okinawa islands were sown in Petri dishes containing filter paper soaked in tap water under darkness at 21 °C for 3 days. Afterwards, the seedlings of vernalized groups were transplanted into plastic
pots and placed inside a growth chamber for vernalization (5 °C, 16-h light/8-h dark) for 4 weeks. At the end of cold treatment, the non-vernalized and vernalized groups were moved together into the same growth chamber at 21 °C, 16-h light/8-h dark. Fresh leaves proximal to the shoot apex were collected from three individuals every 4 h after the lights were turned on (ZT0, Zeitgeber time 0) at the 11-day and 22-day growth stages. Herein, ZT represents a standardized 24-h notation of the phase in an entrained circadian cycle in which ZT0 indicates the beginning of the day or light phase and ZT16 (16 h after light on) refers to the time at which the light-to-dark transition occurs. The 11-day/22-day of growth stages were recorded from the days for planting in the growth chamber for not vernalized group/the end of the cold treatment for vernalized group.

As floral induction genes exhibit the highest expression before bolting (Wang et al. 2018), we only harvested the vernalized southern group at the 11-day growth stage (just before bolting), while the other three groups (i.e. non-vernalized southern, vernalized northern and non-vernalized northern) were analysed at the 11-day and 22-day growth stages. Immediately after harvesting, the samples were flash-frozen in liquid nitrogen. RNA was extracted using the RNasy plant mini kit (Qiagen GmbH, Hilden, Germany) in combination with DNase treatment using the RNase-free DNase Set (Qiagen GmbH) according to the manufacturer’s instructions. In total, 1 μg of RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen GmbH).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR Premix Ex Taq II (including Taq DNA polymerase, reaction buffer and deoxyribonucleotide triphosphate mixture) (Takara Bio Inc.). Each reaction was carried out in a total volume of 20 μL, consisting of 10 μL of SYBR Premix, 2 μL of cDNA sample (diluted 1:3) and 0.4 μM of the forward and reverse primers. qRT-PCR was performed at 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 1 min and a melting curve program of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Constitutively expressed Rs-UBQ was used as a reference gene for normalization (Ayarpadikannan et al. 2014). Gene expression was quantified using the ΔΔCt method (Livak and Schmittgen 2001).

Results

Variation in flowering time

To verify different vernalization requirements (obligate and facultative), we investigated variations in the flowering time of wild radish from Hokkaido and Okinawa islands under long- and short-day light conditions (Fig. 2). Student’s t-test results showed significant differences in flowering time between the vernalized and non-vernalized treatments (P < 0.05). With respect to the northern accessions, under long-day photoperiod treatment with vernalization, flowering onset ranged from 40 to 92 days (mean, 54.3; median, 51); without vernalization, however, regardless of long- or short-day light conditions, flowering failed to occur by 120 days after planting [see Supporting Information— Fig. S1]. In contrast, the vernalized southern accessions flowered significantly earlier (17–21 days; mean, 18.6; median, 19) than the non-vernalized samples (40–67 days; mean, 52.3; median, 52).

Interestingly, the southern lineage was able to flower without cold exposure. Significant differences in flowering time were found between northern and southern groups within the same treatment. Patterns of responses between short- and long-day light treatment were similar, but not statistically significant (P = 0.277 > 0.1) (Fig. 2). Additionally, we found an increasing linear relationship between the number of leaves and number of days from planting to flowering [see Supporting Information—Fig. S2]. Linear fitting analysis showed that R. sativus exhibited multi-leaves with late flowering and sparse leaves with early flowering. The positive correlation indicated the north–south accessions were in the roughly consistent growth rates in vegetative phase until they flowered. Taken together, the southern accessions displayed facultative vernalization requirements while northern accessions were obligate type, consistent with previous study (Han et al. 2016).

To explore the environmental cues that may act as natural selective forces shaping variation in flowering time, we analysed major climatic factors, such as temperature and photoperiod, on the Hokkaido and Okinawa islands [see Supporting Information—Table S1 and Fig. S3]. Mean temperature, daily maximum temperature, daily minimum temperature sunshine duration and day length differed greatly between the northern and southern regions. Field surveys conducted under natural conditions indicated that the southern population germinated in the fall and flowering was delayed until the following spring, whereas the northern population received vernalization of imbibed seed in winter, germinated in spring and flowered in the summer of the same year. These different life cycles may allow wild radish to adapt to seasonal and environmental variations.

Expressions levels of flowering-time-related genes

To test the effect of photoperiod on floral induction, we separated all the treatments into two groups (with long-day or short-day light condition) and performed the Student’s t-test. Results showed no significant differences in flowering time between the two groups (Fig. 2). In addition, considering R. sativus is a facultative long-day light plant, we investigated the responses of gene expression to long-day light (closest to natural conditions) with vernalized and non-ernalized treatments to explore the associations of RsFLC and RsCOLS (A&B) expression and flowering regulation in Japanese wild radish.
Figure 3. Relative expression levels of RsFLC, RsCOL5-A, RsCOL5-B and RsFT in northern and southern accessions of wild radish at the 11-day (left: A–D) and 22-day (right: E–H) growth stages. Black and white bars at the bottom indicate light (16 h) and dark (8 h) phases, respectively (ZT, Zeitgeber time). The error bars represent standard errors of three biological replicates, which are the mean values of three technical replicates.
In the vernalized southern accessions, the expression of RsFLC was reduced after vernalization and was almost undetectable (Fig. 3A). In contrast, the expression levels of RsCOLS-A and RsCOLS-B increased from ZT4, peaked at ZT8 and then decreased to a near-zero level (Fig. 3B and C). RsFT was strongly activated at the end of the photoperiod (ZT16) but was repressed at other times of the day (Fig. 3D). Notably, the kinetic expression of RsFT was possibly related to the abundance of RsCOLS-A and RsCOLS-B. Furthermore, the expression of RsCOLS (A&B) mRNA may result in an abundance of RsFT, a key flowering-time integrator, which contributed to the early onset of flowering. Thereby, genes known to mediate the flowering response to photoperiod in A. thaliana may be also regulated the flowering-time response to vernalization in wild radish.

In the non-vernalized southern accessions, both the abundance and transcriptional activity of RsFLC were high throughout the experiment (Fig. 3A and E). FLC delays flowering by repressing the expression of FT genes involved in floral induction (Searle et al. 2006; Turck et al. 2008). Thus, one could speculate that the observed low expression of RsFT was probably generated by the repression of RsFLC (Fig. 3D). In addition, RsCOLS-B remained downregulated through all stages in the non-vernalized northern accessions. In contrast, the expression of RsCOLS-A was strongly detected at the 11-day growth stage. Furthermore, RsFT expression was high at the 22-day growth stage and peaked at ZT16, accompanied by high RsFLC transcript levels. The high expression of RsFT at 22 days maybe due to the presence of RsCOLS-A activity. It is possible that RsCOLS-A alone was not sufficient to induce flowering when RsFLC was highly expressed at the 11-day growth stage.

In the vernalized northern accessions, the RsFLC mRNA levels were greatly reduced by cold exposure (Fig. 3), in agreement with the findings of Alexandre and Hennig (2008). During the experimental period, RsCOLS-A was expressed at a low level at most time points. In contrast, at the 11-day growth stage, RsCOLS-B increased dramatically from ZT4 and peaked at ZT8. It is assumed that flowering is promoted when plants pass through an accumulation phase to reach a sufficient FT expression level. Interestingly, the peaks in RsFT expression in the vernalized northern population at 22-day overlapped perfectly with the vernalized southern population at 11-day. As overexpression of COLS can induce FT in Arabidopsis (Hassidim et al. 2009), the high RsCOLS-B expression, rather than RsCOLS-A expression, may be correlated with the elevation in RsFT mRNA levels. Flowering in the vernalized northern accessions was plausibly influenced by an increase in the expression of floral promoter RsCOLS-B at the 11-day growth stage and a reduction in the expression of floral repressor RsFLC.

In the non-vernalized northern accessions, RsFLC was highly expressed at the 22-day growth stage (Fig. 3E) and generally exhibited broad peaks at ZT4. Expression levels continued to increase from ZT8, reaching another peak at the end of the dark period (ZT24) at 22 days. Both RsCOLS-A and RsCOLS-B were poorly expressed at the 11-day and 22-day growth stages. As the plants grew, RsFT was nearly completely repressed and was undetectable at most time points, consistent with a potential mechanism in which is strongly suppressed by RsFLC and correlated with RsCOLS-A and RsCOLS-B reduction. Interestingly, in this way, RsFLC would be repressing RsFT expression and thus preventing flowering in the non-vernalized northern accessions. These observations provided hints that the increased RsFLC and the downregulation of RsCOLS-A and RsCOLS-B collectively assisted in the maintenance of a stable downregulated of the floral transition of the RsFT gene, ultimately resulting in vegetative growth without flowering.

**Discussion**

**Differential vernalization requirements of wild radish**

Flowering time is sensitive to climatic signals, including prolonged cold (vernalization) and day length (photoperiod), which serves as ecological cues to ensure that reproductive effort occurs under optimal seasonal environments. Thus, natural variation in flowering may reflect local adaptation to environmental cues. In this research, we investigated natural variation in flowering time of wild radish and the role of two major climatic cues (cold exposure and day length) in regulating flowering transition. In the cultivation experiment, almost the entire northern population remained in the vegetative stage throughout the 120-day growth period, with no signs of floral transition unless exposed to prolonged cold. Conversely, flowering took place in the southern accessions regardless of cold/light treatment, but vernalization significantly promoted flowering time. The southern lineage flowered significantly earlier under cold exposure, but still flowered without vernalization. Thus, vernalization was not required for flowering in the southern group, although it substantially shortened the timing of flowering. These observations confirmed the obligate and facultative vernalization requirements of wild radish, consistent with our previous study (Han et al. 2016). The different requirements and responses to vernalization between the northern and southern populations may reflect the action of different selective pressures shaped by their local habitats.

**Correlation between RsFLC and RsCOLS (A&B) expression patterns**

Genetic analysis of A. thaliana has identified numerous pathways that control the timing of floral transition (Irish 2010). Photoperiod is a major seasonal cue for flowering in A. thaliana and studies have demonstrated that vernalization can overcome the obstruction of photoperiod floral induction (Engelmann and Purugganan 2006). These two flowering pathways (i.e. vernalization and photoperiod) initiate various floral pathway integrator genes, e.g. FT and SOCI (Suppressor of overexpression of CO1), to trigger floral transition (Song et al. 2015). The major role of FLC in the vernalization pathway is to repress flowering by inhibiting the expression of FT and SOCI. Vernalization leads to reduced FLC mRNA and protein levels, thereby removing the FLC-mediated repression of flowering. Hence, exposure to prolonged periods of cold is opposite to the function of the long-day light floral promoter, CO, which activates floral pathway integrator genes.

The molecular basis of the opposing effects of FLC and CO (i.e. vernalization and photoperiod pathways) has received increasing attention (Hepworth et al. 2002; Ream et al. 2014). For instance, overexpression of FLC blocks the activation of SOCI by CO (Hepworth et al. 2002). In addition, high levels of FLC can cause negative regulation of CRY2 (Cryptochrome 2), thereby preventing CRY2 from promoting of CO expression (El-Assal et al. 2003). Consistently, we identified found opposite activity between RsFLC and RsCOLS (A&B) in the regulation of flowering time in the northern and southern accessions. In the northern accessions, flowering in the non-vernalized group was primarily
affected by RsFLC: i.e. overexpression of RsFLC repressed floral initiation, whereas the absence of RsFLC allowed RsCOL5-B to promote flowering after vernalization. In the southern accessions, following the accumulation of RsCOL5-A over time, RsFT increased rapidly, and flowering was successful, despite the action of RsFLC as a floral ‘brake’. Thus, the promoting function of RsCOL5-A may have been stronger than the floral repressor function of RsFLC over time, resulting in sufficient expression of RsFT. Thus, cold exposure and high RsCOL5-A and RsCOL5-B expression may have collectively resulted in significantly earlier flowering times. In contrast, without cold exposure, flowering time was promoted by the overexpression of RsCOL5-A, despite high RsFLC transcript levels. However, the mechanism of RsCOL5-A in upregulating RsFT remains unclear, and further studies using mutants and overexpression lines are required.

In addition, the expression patterns did not provide evidence for interactions among all genes; thus, further genetic studies and in vivo experiments are needed to verify protein–protein or protein–DNA interactions.

As the southern populations did not require vernalization, we studied the molecular mechanism underlying early flowering in these plants. Summer-annual strains of Arabidopsis consistently display low FLC expression to promote flowering, resulting from the absence of FRI activity (Johanson et al. 2006) or disruption of the FLC regulatory sequences by transposons (Michaels et al. 2003). In wild radish, however, high RsFT activity and the consistent early flowering phenotype implied that RsCOL5-A may initiate flowering in the presence of the floral repressor RsFLC. Many studies have reported that the vernalization pathway can override other floral pathways (Tadege et al. 2003; Alexandre and Hennig 2008; Miller et al. 2008). In our work, however, RsCOL5-A in the southern accessions induced flowering, even under a background of RsFLC expression, indicating a preference for the photoperiod pathway rather than the vernalization pathway. However, further studies are required to explore the specific molecular mechanisms underlying the dominance of the photoperiod pathway in non-vernalized southern populations of Japanese wild radish, i.e. the floral promotion of RsCOL5-A under high RsFLC expression. Such research will offer new hints on the molecular mechanisms underpinning adaptive variation in flowering time.

Natural selection may affect floral pathway dominance

Wild radish is a genetically diverse and highly adaptable species that thrives in a wide range of environments (Madhou et al. 2005). The daily minimum temperature from October to February ranged from 14.6 to 23.1 °C in Okinawa (Naha), but from −7 to 7.5 °C in Hokkaido (Sapporo) [see Supporting Information—Table S1]. Considering that the optimum vernalization temperature ranges from 1 to 7 °C in most species (Amasino 2010; Duncan et al. 2015) and the daily minimum temperature in the southern region is higher than the favourable vernalization temperature, it is plausible that the southern accessions were unable to undergo vernalization. In contrast, the northern temperatures are cold enough for vernalization to initiate flowering in the northern populations. These observations confirm the different vernalization requirements of the northern and southern accessions (i.e. obligate and facultative, respectively) in agreement with earlier research (Han et al. 2016). Based on field investigation, the day length required for flowering was much shorter for the southern population than for the northern population under field conditions. Specifically, the southern population tended to favour short-day light condition (−11 h in February; see Supporting Information—Fig. S3), whereas the northern accessions preferred long-day light condition (−14.5 h in May; see Supporting Information—Fig. S3) after prolonged cold exposure to initiate flowering. These results imply that differences in dominance of floral pathways may be generated by natural selection.

Flowering time is an important determinant of fitness in a variable environment and responds plastically to seasonal cues involving temperature and photoperiod (Amasino 2010). Based on field surveys, we found that the adaptive strategies of Japanese wild radish were nearly consistent with the winter-summer annual ecotypes of A. thaliana covering a range of latitudes (Engelmann and Purugganan 2006; Vidigal et al. 2016). Specifically, the southern accessions, like the winter-annual ecotype of A. thaliana, germinated in the autumn and overwintered as rosettes, where they experienced short-day lengths, and then flowered in the following spring. In contrast, northern samples germinated in spring and flowered and set seed in the same summer or autumn season, consistent with the summer-annual ecotype of A. thaliana. However, unlike the winter-annual Arabidopsis ecotype, the southern lineage of Japanese wild radish did not require vernalization to trigger flowering. In contrast, although the northern wild radish samples resembled summer-annual of Arabidopsis, they needed vernalization to promote floral transition. The different life-history traits control the time invested in vegetative growth and time to reproduction, reflecting adaptations of the life cycle to the broad environmental and ecological diversity.

Conclusions

Flowering time is a developmental transition that is sensitive to ecological cues and can adapt to seasonal and environmental variations. Our research showed that the RsFLC and RsCOL5 (A&B) genes interact to regulate flowering time and floral pathway dominance is affected by their seasonal cues. Notably, the northern accessions may modulate flowering time primarily through the vernalization pathway, whereas the southern accessions may preferentially mediate flowering time based on the photoperiod pathway under non-vernalization conditions. Thus, these mechanisms help initiate optimal flowering time in natural populations in response to local climatic cues. Our results indicate rich natural diversity in photoperiod and vernalization requirements to exploit for understanding gene networks controlling flowering, which will offer new hints about the molecular mechanism underpinning adaptive variation of flowering time, adapting to ongoing and future climate change. However, further studies are required to understand how plants alter flowering pathway activity to adapt to growth in different geographical locations and how plants balance the effects of different environmental stimuli on flowering time at a more precise molecular level, which is important for the efficient manipulation of flowering in crop production.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. The monthly/annual climate normal for major observatories in Sapporo and Naha in Japan, calculated from 1981 to 2010.

Table S2. Locus information and list of the primers for gene expression analyses.
**Figure S1.** Vegetative growing and flowering ones at 60-day (A) and 120-day (B) after the wild radish was planted in the greenhouse. In the respective picture, it displayed not-flowering (left) of non-vernalized northern individual and flowering (right) individuals from the southern region.

**Figure S2.** Leave number versus flowering time and its linear fitting approach.

**Figure S3.** Mean day length per month of the studied locations. Day length: the number of hours when sun is above the horizon line.

**Sources of Funding**
This research was funded by grants from the Grants-in-Aid for Scientific Research of Japan (No. 16H04831/SPS KAKENHI and 24247013/SPS KAKENHI), the National Natural Science Foundation of China (31700188), Shandong Provincial Natural Science Foundation of China (ZR2017QZ16323) and Excellent Youth Innovation Team of University in Shandong Province, China (2019KJE020 and 2020KJE008).

**Contributions by the Authors**
Q.X.H. and T.W. surveyed the populations in the field and collected the tissue for laboratory work. Q.X.H. performed experiments and analysed the sequence data and wrote most of the manuscript. S.S. revised the manuscript. H.S. supervised the project design, laboratory work and analyses. All authors read and approved the final manuscript.

**Conflict of Interest**
None declared.

**Acknowledgements**
We thank Dr. Daiki Takahashi and Shinichiro Kameoka (Kyoto University) for assisting in field survey and sample collection. We thank Dr. Yuki Mitsui (Tokyo University of Agriculture) for providing us genome informations and the valuable comments on our manuscript.

**Data Availability**
Data and gene sequences used in this paper are available on Figshare: https://figshare.com/s/4be0a586c85899a7e5a3.

**Literature Cited**
Alexandre CM, Hennig L. 2008. FLC or not FLC: the other side of vernalization. *Journal of Experimental Botany* 59:1127–1135.

Amasino RM. 2010. Seasonal and developmental timing of flowering. *Plant Journal* 61:1001–1013.

Ayapuddikannan S, Chung E, Kim K, So HA, Schrauwenfle KR, Lee JH. 2014. RsERF1 derived from wild radish (*Raphanus sativus*) confers salt stress tolerance in Arabidopsis. *Acta Physiologiae Plantarum* 36:993–1008.

Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O. 2006. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science* 312:1040–1043.

Chen J, Chen JY, Wang JN, Kuang JF, Shan W, Lu WJ. 2012. Molecular characterization and expression profiles of MaCOL1, a CONSTANS-like gene in banana fruit. *Gene* 496:110–117.

Cheng XF, Wang ZY. 2005. Overexpression of COL9, a CONSTANS-LIKE gene, delays flowering by reducing expression of CO and FT in Arabidopsis thaliana. *The Plant Journal* 43:758–768.

Cho LH, Yoon J, An G. 2017. The control of flowering time by environmental factors. *The Plant Journal* 90:708–719.

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gislot L, Turnbull C. 2007. FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316:1030–1033.

Datta S, Hettiarachchi GH, Deng XW, Holm M. 2006. Arabidopsis CONSTANS-LIKE3 is a positive regulator of red light signaling and root growth. *The Plant Cell* 18:70–84.

Deng W, Ying H, Heliwell CA, Taylor JM, Peacock WJ, Dennis ES. 2011. *FLOWERING LOCUS C* (FLC) regulates development pathways throughout the life cycle of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 108:6680–6685.

Dowovan BC, Koch MA, Michael M, Klaus M, O’Kane SL, Warwick SI, Windham MD, Al-Shehbaz IA. 2006. Toward a global phylogeny of the Brassicaceae. *Molecular Biology & Evolution* 23:2142–2160.

Duncan S, Holm S, Questa J, Irwin JA, Grant A., Dean C. 2015. Seasonal shift in timing of vernalization as an adaptation to extreme winter. *eLife* 4:e06620.

El-Assal SED, Alonso-Blanco C, Peeters AJ, Wagemaker C, Weller JL, Koornneef M. 2003. The role of cryptochrome 2 in flowering in *Arabidopsis*. *Plant Physiology* 133:1504–1516.

Engelmann K, Purugganan M. 2006. The molecular evolutionary ecology of plant development: flowering time in Arabidopsis thaliana. *Advances in Botanical Research* 44:507–526.

Fitter A, Fitter R. 2002. Rapid changes in flowering time in British plants. *Science* 296:1689–1691.

Fornara F, Montaigu AD, Coupland G. 2010. SnapShot: control of flowering in *Arabidopsis*. *Cell* 141:550.e1-2.

Han Q, Higashi H, Mitsui Y, Setoguchi H. 2016. Lineage isolation in the face of active gene flow in the coastal plant wild radish is reinforced by differentiated vernalisation responses. *BMC Evolutionary Biology* 16:94.

Hassidim M, Harir Y, Yakir E, Kron I, Green RM. 2009. Over-expression of CONSTANS-LIKE 5 can induce flowering in short-day grown Arabidopsis. *Planta* 230:481–491.

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G. 2002. Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *The EMBO Journal* 21:4327–4337.

Hu T, Wei Q, Wang W, Hu H, Bao C. 2018. Genome-wide identification and characterization of CONSTANS-like gene family in radish (*Raphanus sativus*). PLoS ONE 13:e0204137.

Huijser P, Schmid M. 2011. The control of developmental phase transitions in plants. *Development* 138:4117–4129.

Irish V. 2010. The flowering of *Arabidopsis* flower development. *Plant Journal for Cell & Molecular Biology* 61:1014–1028.

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C. 2000. Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. *Science* 290:344–347.

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D. 1999. Activation tagging of the floral inducer FT. *Science* 286:1962–1965.

Kim-month-Sultz HA, Tong X, Lee J, Song YH, Ito S, Kim SH, Imaizumi T. 2016. Cool night-time temperatures induce the expression of CONSTANS and FLOWERING LOCUS T to regulate flowering in Arabidopsis. *The New Phytologist* 211:208–224.

Kitashiba H, Yokoi S. 2017. Genes for bolting and flowering. In: The radish genome. Cham, Switzerland: Springer, 151–163.

Komedá Y. 2004. Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annual Review of Plant Biology* 55:521–535.

Ledger S, Strayer C, Ashton F, Kay SA, Futteril J. 2001. Analysis of the function of two circadian-regulated CONSTANS-LIKE genes. *The Plant Journal* 26:15–22.

Lee JH, Kim YC, Jung Y, Han JH, Zhang C, Yun CW, Lee S. 2019. The overexpression of cucumber (*Cucumis sativus*) L. genes that encode the branched-chain amino acid transerase modulate flowering time in *Arabidopsis thaliana*. *Plant Cell Reports* 38:25–35.

Li Y, Huang Y, Bergelson J, Nordborg M, Borevitz JO. 2010. Association mapping of local climate-sensitive quantitative trait loci in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 107:21199–21204.
Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. Methods 25:402–408.

Madhou P, Wells A, Pang E, Stevenson T. 2005. Genetic variation in populations of Western Australian wild radish. Australian Journal of Agricultural Research 56:1079–1087.

Michaels SD, Amasino RM. 2000. Memories of winter: vernalization and the competence to flower. Plant, Cell & Environment 23:1145–1153.

Michaels SD, Amasino RM. 2001. Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. The Plant Cell 13:935–941.

Müller TA, Muslin EH, Dorweiler JE. 2008. A maize CONSTANS-like gene, conz1, exhibits distinct diurnal expression patterns in varied photoperiods. Planta 227:1377–1388.

Nie S, Li C, Xu L, Wang Y, Huang D, Muleke EM, Sun X, Xie Y, Liu L. 2016. De novo transcriptome analysis in radish (Raphanus sativus L.) and identification of critical genes involved in bolting and flowering. BMC Genomics 17:389.

Puterill J, Laurie R, Macknight R. 2004. It’s time to flower: the genetic control of flowering time. Bioessays 26:363–373.

Raman H, Raman R, Coombes N, Song J, Prangnell R, Bandaranayake C, Tahira R, Sundaramoorthy V, Killian A, Meng J. 2016. Genome-wide association analyses reveal complex genetic architecture underlying natural variation for flowering time in canola. Plant, Cell & Environment 39:1228–1239.

Ream TS, Woods DP, Schwartz CJ, Sanabria CP, Mahoy JA, Walters EM, Kaeppler HF, Amasino RM. 2014. Interaction of photoperiod and vernalization determines flowering time of Brachypodium distachyon. Plant Physiology 164:694–709.

Robson F, Costa MMR, Hepworth SR, Vizir I, Pineiro M, Reeves PH, Putterill J, Coupland G. 2001. Functional importance of conserved domains in the flowering-time gene FLC. Plant, Cell & Environment 24:2303.

Satake A, Kawagoe T, Saburi Y, Chiba Y, Sakurai G, Kudoh H. 2013. Forecasting flowering under climate warming by controlling flowering: time measurement mechanisms in leaves. Annual Review of Plant Biology 64:441–464.

Suárez-López F, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. 2001. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature 410:1116–1120.

Sung S, Amasino RM. 2005. Remembering winter: toward a molecular understanding of vernalization. Annual Review of Plant Biology 56:491–508.

Tadege M, Sheldon CC, Helliwell CA, Upadhyaya NM, Dennis ES, Peacock WJ. 2003. Reciprocal control of flowering time by OsSCO1 in transgenic Arabidopsis and by FLC in transgenic rice. Plant Biotechnology Journal 1:361–369.

Turck F, Fornara F, Coupland G. 2008. Regulation and identity of the flowering time FLOWERING LOCUS T moves center stage. Annual Review of Plant Biology 59:573–594.

Vülügal DS, Marques AC, Willems LA, Buijs G, Méndez-Vigo B, Hilhorst HW, Beutsink L, Picó FX, Alonso-Blanco C. 2016. Alitudinal and climatic associations of seed dormancy and flowering traits evidence adaptation of annual life cycle timing in Arabidopsis thaliana. Plant, Cell & Environment 39:1737–1748.

Wang S, Li Z, Jin W, Fang Y, Yang Q, Xiang J. 2018. Transcriptome analysis and identification of genes associated with flower development in Rhododendron pulchrum Sweet (Ericaceae). Gene 679:108–118.

Wang Q, Zhang L, Zheng P. 2015. Genetic diversity and evolutionary relationship analyses within and among Raphanus species using EST-SSR markers. Molecular Breeding 35:62.

Weber K, Burow M. 2018. Nitrogen - essential macronutrient and signal controlling flowering time. Physiologia Plantarum 162:251–260.

Xiao D, Shen HR, Zhao JJ, Wei YP, Liu DR, Hou XI, Bonnema G. 2019. Genetic dissection of flowering time in Brassica rapa responses to temperature and photoperiod. Plant Science 280:110–119.

Yamagishi H. 2004. Assessment of cytoplasmic polymorphisms by PCR-RFLP of the mitochondrial orfB region in wild and cultivated radishes (Raphanus). Plant Breeding 123:141–144.

Yamane K, Li N, Ohnishi O. 2009. Multiple origins and high genetic diversity of cultivated radish inferred from polymorphism in chloroplast simple sequence repeats. Breeding Science 59:55–65.

Yi G, Park H, Kim JS, Chae WB, Park S, Huh JH. 2014. Identification of three FLOWERING LOCUS C genes responsible for vernalization response in radish (Raphanus sativus L.). Horticulture, Environment and Biotechnology 55:548–556.

Zheng Y, Luo L, Liu Y, Yang Y, Wang C, Kong X, Yang Y. 2018. Effect of vernalization on tuberization and flowering in the Tibetan turnip is associated with changes in the expression of FLC homologues. Plant Diversity 40:50–56.