Natural variation of flowering time and its genetic basis in Arabidopsis kamchatica subsp. Kawasakiana

杉阪，次郎

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神戸大学博士論文

タチスズシロソウにおける開花タイミングの自然変異とその遺伝的基礎

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Chapter 1

General introduction

Exploring the genetic basis of adaptation has become one of the central tasks of evolutionary ecology. Knowledge on genetic architecture of adaptive traits allows us to identify genes involved in adaptation and speciation (Mitchell-Olds 2001; Shimizu 2002; Gazzani et al. 2003). Rapid advance of genomics in the last decade has facilitated ecologists and molecular biologists to address fundamental questions in evolutionary biology with genomic approaches, establishing a new discipline often called as "Ecological genomics" (Shimizu and Purugganan 2005).

Phenotypic responses to environmental fluctuations are critical for sessile organisms, such as plants, in adaptation to natural habitats. Phenotypic plasticity is the ability of a single genotype to produce series of phenotypes depending on developmental environments, and is considered to be ubiquitous among living organisms (Bradshaw 1965; Schlichting 1986; Sultan 1987; Pigliucci 2006). Ecological study of phenotypic plasticity have been conducted by the two following approaches: (1) observation of phenotypic change in response to environmental cues either in a natural populations or under laboratory conditions, (2) estimating the adaptive value of phenotypic plasticity by measuring fitness in variable environments. However the genetic basis of phenotypic plasticity is still largely unknown and we even have to answer very basic question, such as what kind of and how many genes are involved phenotypic plasticity (Pigliucci 2001).

In the model organisms *Arabidopsis thaliana* (L.) Heynh, phenotypic plasticity of some ecologically important traits has been dissected genetically (Johanson et al. 2000; Michaels et al. 2003; Caicedo et al. 2004). Among them, flowering regulatory is the most extensively studied system in its genetics mechanisms of environmental responses (Sung and Amasino 2004a). Number of genes has been identified to respond to environmental cues such as temperature and photoperiod (Jhonson et al. 2000; Sheldon et al. 2000; Michaels and Amasino 2001; Simpson and Dean 2002; Michaels et al. 2003; Henderson and Dean 2004; Putterill et al. 2004). More importantly, we need to know how these genes in the regulatory system produce natural variation of flowering time, because genetic variation is a foundamental source of adaptative evolution (Alonso-Blanco and Koornneef 2000; Michell-Olds 2001; Shimizu 2002; Shimizu and Purugganan 2005; Lempe et al. 2005; Tonsor et al. 2005; Michell-Olds and Schmitt 2006).

In this thesis, I studied the ecology of flowering time and its response to prolonged cold temperatures in natural populations of *Arabidopsis kamchatica* (Fisch. ex DC.) K. Shimizu & Kudoh subsp. *kawasakiana* (Makino) K. Shimizu & Kudoh. Natural variation of the flowering responses along a latitudinal gradient was examined and its genetic basis was explored using the approach of ecological genomics. Because the study species is a close relative of A. thaliana, we predicted the candidate gene to be studied based on the knowledge on the regulatory mechanisms of flowering time in the model species. In this study, I focused on the role of variation in *FLC* expression in explaining the observed variation in flowering time.

A typical life cycle of major ecotypes of *A. thaliana* is a winter annual, i.e., seeds germinate in autumn, and plants over-winter as a rosette, and reproduce in early spring. Reproduction in spring initiate by bolting of flowering stalks followed by flowering and seed set, and plants wither after reproduction. Temperature and photoperiod are considered to be two major environmental cues that control flowering time in spring. Following genes are known to have a central role in the responses to these environmental cues.

FLC

FLOWERING LOCUS C (*FLC*) plays a central role in controlling flowering time through vernalization, i.e, exposure to prolonged cold temperature during winter) (Michaels and Amasino 1999; Sheldon et al. 2000). *FLC* is a transcription factor and a member of MADS-box gene family. In the winter annual strains of *A. thaliana*, a high level of *FLC* is expressed in seedling to juvenile stage, then transition to flowering is suppressed for longer periods during successive vegetative growth (Michaels and Amasino 1999). Prolonged cold temperature is known to suppress the expression level of *FLC*, and trigger flowering in the following warm temperatures (Sheldon et al. 1999). When the plants are grown without low temperature, it continues to grow vegetatively for a longer period prior to flowering. Several lines of *flc* mutants have been identified, and they usually flower early without vernalization (Michaels et al. 2003).

VRN1, *VRN2*, *VIN3*

Several genes are involved in the temperature-mediated winter suppression of *FLC*. *VERNALIZATION1* (*VRN1*), *VRN2* and *VERNALIZATION INSENSITIVE3* (*VIN3*) are known to be necessary in the response to low temperature (Levy et al. 2002; Sung and Amasino 2004a, 2005). In *vrn1* and *vrn2* mutants, *FLC* expression is reduced when grown under low temperature (Gandell et al. 2001, Levy et al 2002). However, *FLC* expression is restored soon after the cease of cold treatments. This suggests that *VRN1* and *VRN2* are involved in the stabilized suppression of *FLC* after vernalization. In the *vin3* mutants, *FLC* is mot suppressed by low temperature. This indicated that *VIN3* is required for the initial suppression of *FLC*. It has been reported that the expression level of *VIN3* increase in response to cold temperature (Sung and Amasino 2004b).

FRI

FRIGIDA (*FRI*) gene is known to up-regulate *FLC* expression and serves as a mechanism to prevent flowering before winter (Johanson et al 2000; Le Corre at al. 2002; Shindo et al. 2005). The *fri* mutant shows early flowering phenotype, i.e. it flowers early even without vernalization (Johanson et al. 2000). Some early-flowering ecotypes including Col are known to have nonfunctional *FRI* alleles. Natural variation in *FRI* has been reported, and the epistasis between *FRI* and *FLC* is thought to be involved in the formation of latitudinal clines of flowering time among natural population (Jhonson et al. 2000).

Recent studies revealed that *FLC* and *FRI* is responsible for the variation in flowering

time, its plasticity to environmental factors, and variation in life cycles in *A. thaliana* (Grazzani et al. 2003; but see Shindo et al. 2005; Werner et al. 2005). For example, loss-of-function mutations in *FLC* or *FRI* result in early-flowering ecotypes that behave as summer annuals in natural conditions (Johnson et al. 2000, Michaels et al. 2003). Molecular comparisons of alleles of these loci indicate that the evolution of summer annuals caused by *FRI* and *FLC* mutations has occurred multiple times in the lineages of *A. thaliana* (Le Corres et al. 2002; Michaels et al. 2003).

Genomic study in *A. thaliana*, such as described above, made it possible to study the genetic basis of adaptive evolution in natural populations. Especially, close relatives of *A. thaliana* are suitable for addressing ecological and evolutionary issues simultaneously by applying molecular genetics approaches. On the other hand, basic ecology of wild *Arabidopsis* species, such as distribution, phenology, habitat, life history and breeding system, are poorly known to date. The study species, *A. kamchatica* subsp. *kawasakiana*, is a close relative of *A. thaliana*, and thus allowed the use of molecular genetic approach in the study of natural variation and adaptive evolution of flowering time. In addition, *A. kamchatica* subsp. *kawasakiana* is allotetraploid originating from hybridization of *A. lyrata* and *A. halleri* subsp. *gemmifera*. Therefore I also can address questions concerning the genetic effects of polyploidization on flowering time regulation in natural populations.

The goal of this study were (1) to describe basic ecological characteristics of A. kamchatica subsp. kawasakiana, and (2) to clarify the genetic basis of natural variation in flowering time and its plasticity in the study species and (3) to explore how variation

in FLC alleles contributes natural variation in flowering time.

First, I studied basic ecological characteristics of the study species, such as distribution range, life history, population structure, and breeding systems (Chapter 2). Second, I examined natural variation in flowering time plasticity, i.e. flowering-time responses to different length of cold treatment, in a growth experiment (Chapter 3). Third, I studied the plasticity of *FLC* expression in response to vernalization and its variation among populations. I also explore the correlation between *FLC* expression and flowering time variation by dissecting the *FLC* expression into *FLC* copies of different parental origin (Chapter 4). Finally, significance of my results in understanding genetic basis of natural variation is discussed (Chapter 5).

Chapter 2

Phenology and breeding system in natural population of *Arabidopsis kamchatica* subsp. *kawasakiana*

Abstract

Distribution, local population structure, life cycle and the breeding system of *Arabidopsis kamchatica* subsp. *kawasakiana* (Brassicaceae) were studied and described here. The taxon occurs sporadically in the Hokuriku, Kinki, and Tokai districts in western Honshu and Shikoku and grows along sandy sea and lake shore. Majority of the local populations of this subspecies have become extinct because of habitat loss by human activities that altered shore environments. To investigate local population structure and life cycle schedule of *A. kamchatica* subsp. *kawasakiana*, we conducted a set of field studies in the largest population at Nakasho-hama, Makino-cho, Takashima-shi, Shiga Prefecture. This species showed life cycles as a typical winter annual, and occurs in narrow belts along boundaries between perennial vegetation and sandy shore. Furthermore, the breeding system of this species was studied in three natural populations. I applied four experimental treatments, open pollination, bagging, emasculation + bagging and emasculation + hand-pollinated + bagging. None of the emasculated flowers with bags produced fruits but we observed high fruit sets in the other three treatments. The results confirmed that *A. kamchatica* subsp. *kawasakiana* is a self-compatible, non-apomictics species that can produce seeds through auto-pollination. Considering the life cycle as an annual, increased reproductive

assurance through auto-pollination should be critical for the maintenance of populations.

Introduction

Wild relatives of a model plant, *Arabidopsis thaliana*, have recently become to draw attentions of botanist of diverse disciplines from molecular biology to evolutionary ecology (Mitchell-Olds 2001; Clauss and Koch 2006). The study of these species allows the use of enormous amount of information from the genome analysis of the model plant. The study of the wild *Arabidopsis* provides the opportunity to study genetic backgrounds of novel phenotypes that are not observed in the model species. Furthermore, it gives the opportunity to study genetic bases of natural variation of ecologically interesting plant traits.

As a result of recent analyses in molecular phylogenetics, several Japanese taxa that had been considered as members of the genus *Arabis* were transferred to the genus *Arabidopsis* (O'Kane & Al-Shehbaz 1997). The study species, *Arabidopsis kamchatica* subsp. *kawasakiana* (Fig. 2-1), is one of the plants that have turned to be close relatives of *Arabidopsis thaliana*. Contrary to the increasing attention, field study of wild *Arabidopsis* is limited and its distribution, ecology and life history is still largely unknown (Clauss and Koch 2006).

Arabidopsis kamchatica subsp. *kawasakiana* is an annual that occurs along sandy sea and lake shores of western Japan (Fig. 2-2). It probably derived from the closely related subspecies, *A. kamchatica* subsp. *kamchatica* that has perennial life history and wider

range of distribution. The both subspecies are allotetraploid (Shimizu et al. 2005), and provide opportunity to study the role of genome duplication in plant evolution (Madlung et al. 2002). *A.kamchatica* subsp. *kawasakiana* is now endangered and its ecology needs to be studied both for its conservation of natural populations and of resources for plant evolutionary study.

The aim of the study is first to reveal current distribution and life history of this endangered species, and second to reveal its breeding systems in the natural populations.

Materials & Methods

Plants

Arabidopsis kamchatica (Ledeb.) K. Shimizu & Kudoh subsp. *kawasakiana* (Makino) K. Shimizu & Kudoh grows on sandy sea /lake shores of western Honshu, Japan. The taxon was originally described as *Arabis kawasakiana* Makino (Makino 1913). The type specimen of the taxon (lectotype) is designed by Shimizu et al. (2005) and preserved in MAK (the Makino Herbarium, Tokyo Metropolitan University, Fig. 2-3). It became treated as a member of *Arabidopsis* based on the molecular phylogenetic studies of the genus (O'Kane & Al-Shebaz 1997). Although the taxon is tetraploid ($2n = 4x = 32$), it had been considered to be closely related with a Eurasian and North American self-incompatible diploid, *Arabidopsis lyrata* Kitamura. (O'Kane & Al-Shebaz 1997). Recently, it turned out that the taxa share their allotetraploid origins (expected parental taxa are *A. lyrata* and *A. halleri* subsp. *gemmifera*) with *A. kamchatica* subsp.

kamchatica, and then it is now treated as *A. kamchatica* subsp. *kawasakiana* (Shimizu et al. 2005).

Distribution

Specimens deposited in the herbaria of Kyoto University (KYO), Tokyo Metropolitan University (MAK), and Shoei Junior College (Kobe-shi, Japan) were examined and the localities are recorded. Together with information from literatures, the distribution map is constructed. Based on the obtained information, we visited the localities and observed the current occurrence of *A. kamchatica* subsp. *kawasakiana* on site.

Breeding system

To evaluate the breeding system of *A. kamchatica* subsp. *kawasakiana* in natural populations, we examined levels of fruit set in natural populations and conducted sets of field experiments that combined emasculation, hand-pollination and bagging treatments.

The field experiments were conducted in three natural populations that were selected from north, central and south parts of the geographic distribution of the subspecies (Table 2-1). The Hamakurosaki site (Toyama-shi, Toyama Prefecture) is located in a sandy open habitat along the coast of Toyama Bay. The Nakashohama site (Takashima-shi, Shiga Prefecture) is on the western shore of the Lake Biwa, the largest fresh-water lake in s Japan. The Fukiiura site (Matsusaka-shi, Mie Prefecture) is a sandy open site in a coastal pine forest of Ise Bay.

At the Hamakurosaki, Nakashohama and Fukiiura sites, 56, 204 and 36 plants

were used in the experiments, respectively. Neighboring four plants are assigned as a set which were apart from over two meter among sets, and we designated 14, 51 and 9 sets for the three sites, respectively. The sample sizes were determined by the number of flowering individuals on each site. Each of the following four treatments was randomly applied to one of the plants in a set. For each plant, we selected a flower bud that will open in the next day and marked by a string that was tied on a peduncle. In the open pollination treatment, the flower buds were untouched and flowers were open to pollinators. We considered that the fruit sets in the open-pollination treatments represented those observed in natural conditions, and the results of this treatment served as controls with which other treatments were compared statistically. In the bagging treatment, each flower bud was covered with a small plastic bag to prevent pollinators from visiting the flower. Bags had tiny holes for ventilation. We expected high fruit set in the bagging treatment if the plants produce seed either by apomixis or by auto-pollination. In the emasculation + bagging treatment, anthers were removed from the flower buds before its flowering. For emasculation, we removed sepals and petals from the flower buds. The flowers were bagged immediately after the emasculation. If we do not see any fruit set in this treatment, it suggests that the flowers require pollens to set fruit. Then, we can exclude apomictic seed reproduction from the list of possible breeding systems. In the emasculation + hand-pollination + bagging treatments, stigmas of each emasculated flower was hand-pollinated using the pollen grains from a single flower on a plant over five meters away from the treated plant. We set this treatment as a second control to evaluate our experimental procedures. High fruit set in this

treatment would suggest that the damage by bagging and emasculation treatments themselves were not preventing fruit set. The experimental treatments were applied on 10 May, 16-17 April and 18-19 May, and 18 April 2003 for the Hamakurosaki, Nakashohama, and Fukiiura, respectively.

Bags on the flowers were removed 4 - 7 days after the anthesis so that the bags did not interfere with fruit development. I recorded number of flowers that have developed fruits ca. four weeks after the treatments. Fruit set was calculated as the proportion of flower that produced fruits for each treatment within each site. Fruit set in the bagging, emasculation + bagging, and emasculation + hand-pollination + bagging treatments were compared against the open-pollination treatment using the chi square test (Sokall and Rohlf 1995). In these tests, we kept the total error rate $\alpha = 0.05$ and 0.01 by the sequential Bonferoni methods (Rice 1989; Sokall and Rohlf 1995).

I also observed pollinators in the three sites during we conducted experimental treatments.

Local population structure and life cycle

To investigate local population structure and life cycle schedule of *A. kamchatica* subsp. *kawasakiana*, we conducted a set of field studies in the largest population at Nakasho-hama, Makino-cho, Takashima-shi, Shiga Prefecture.

Local distribution pattern of individuals and its fluctuation between years were studied in a 5 x 1,000 m belt-transect set on Apr. 2002. The transect set was divided into 200 5 x 5 m sub-quadrat. Number of individuals in each quadrat was recorded in the

spring for successive two years, i.e., April 13 and 19, 2002 and Apr. 9 and 15, 2003.

At the beginning of September 2002, I set 30 1 m x 1 m quadrats with the following arrangement. A group of three quadrats were set at 5m intervals on a vertical line against lakeshore line, and we set 10 groups of three quadrats at 50 m intervals along 450 m lakeshore. These quadrats were visited at one-week intervals for two growth seasons between Sep. 4, 2002 and Aug. 4, 2004. We recorded number of rosettes and flowering individuals within quadrats to quantify life cycle schedules, especially to identify the timing of seedling emergence, seedling mortality and reproduction. At the seedling stages with cotyledons, it was difficult to identify the species. We recorded individuals after they opened true leaves. Usually it takes 5-10 days to open true leaves after seedling emergence of *A. kamchatica* subsp. *kawasakiana.*

Results

Distribution

Our investigation on herbarium specimens revealed that *A.kamchatica* subsp. *kawasakiana* distributes sporadically Hokuriku, Kinki, and Tokai districts in western Honshu and Shikoku (Fig. 2-4). Field observations, however, showed that *A. kamchatica* subsp. *kawasakiana* have extinct or been in endanger in the majority of the localities. The existent populations occur only in Toyama, Shiga, Mie, and Kochi Prefectures.

We confirmed occurrence of *A. kamchatica* subsp. *kawasakiana* at Hamakurosaki, Toyama-shi (confirmed on Apr., 2003) in Toyama Prefecture: in Shiga

Prefecture, Nakasho-hama, Makino-cho (Apr., 2002), Imazuhama, Imazu-cho (Apr., 2002), Shirahigehama, Takashima-cho (May 2005), Takashima-shi; Ohmimaiko (Apr., 2002), Kitahira (Apr. 2002), Shiga-cho, Shiga-gun; and Satsuma-cho, Hikone-shi (Apr. 2002): Fukiiura, Matsusaka-shi (Apr. 2002) in Mie Prefectur: and Tanesaki, Kochi-shi (Apr. 2004) in Kochi Prefecture.

Among the four prefectures in which we confirmed current occurrence of the taxon, shore of the Lake Biwa in Shiga Prefecture is the only region where we found multiple populations. All of these populations except for those in Nakashohama and Shirahigehama at the Lake Biwa were small in terms of both number of plants and area occupied by plants. Even in the two large populations, due to constructions for shore protection and camping site, the area occupied by *A. kamchatica* subsp. *kawasakiana* was reducing. For other three prefectures, we found single population per prefecture. They all occur in very small sites and contained small number (less than several hundred individuals) of plants. All sites are in local old graveyards (Fig. 2-2b) or near a small shrine, and maintained by infrequent weeding.

Breeding system

In the open-pollination treatment, we observed high fruit set (0.89 - 0.98) at all three sites (Table 2-2). All of the bagged flowers developed fruits and there were no statistical differences between the bagging and the open-pollination treatments at the three sites (Table 2-2). None of the emasculated flower produced fruits and the differences against the open-pollination treatment were highly significant at the all three sites (Table 2-2).

The emasculation + hand-pollination + bagging treatment resulted in the high fruit set (0.86 - 1.00), and no statistical difference were detected compared to the open-pollination treatment at the all sites (Table 2-2).

During the experiments flowers were often visited by a small Coleoptera, *Oedemeronia lucidicollis* Motschulsky (Fig. 2-1b), at the most southern site, Fukiiura. However, we observed few pollinators visiting flowers at the Hamakurosaki and Nakashohama sites.

Local population structure and life cycle

Number of individuals recorded in the 5 x 1,000 m transects was 6,486 and 6,842 in the spring of 2002 and 2003, respectively. The patterns of local distributions were generally similar between 2002 and 2003 (Fig. 2-5 and Fig. 2-6). The plants distributed patchily, and *A. kamchatica* subsp. *kawasakiana* was absent from 129 and 106 5 x 5 m's subquadrats in 2002 and 2003, respectively. Plant densities per 5 x 5 m's subquadrat with *A. kamchatica* ssp. *kawasakiana* were 0.04 – 32.72 and 0.04 – 22.08 /m^2 in 2002 and 2003, respectively. New patch formations in 2003 mostly occurred near the 2002 patches, but there were a few cases of new patch formation in distant areas from the 2002 patches (Fig. 2-7).

Among ten 1 x 1 m quadrats each at lakeside, central and land-side, occurrence of *A. kamchatica* subsp. *kawasakiana* were found in 1, 6, and 1 quadrats, respectively. Maximum number of flowering individuals per quadrat was 4, 66, and 4 for lakeside, central and landside quadrats, respectively (Fig. 2-8). There were no apparent

differences in the timings of seedling emergence and flowering among quadrats. Phenological pattern in a representative quadrat was shown in Fig 2-8. No individuals were observed during summer and the plants showed the life cycle as a typical winter annual (Fig. 2-9). Seedling emergence starts at late October. Most of observed seedling mortality occurred during autumn and few died during winter (Fig. 2-9). Flowering started in the middle of April, and all individuals were flowered by late June (Fig. 2-9). All these flowering individuals withered by early in July (Fig. 2-9).

Discussion

Herbarium specimens and literatures suggested that *A. kamchatica* subsp. *kawasakiana* distributed at least in nine prefectures of western Honshu and Shikoku, i.e. Shizuoka, Toyama, Aichi, Mie, Osaka, Hyogo, Ehime and Kochi, until 1920's. Field observations, however, showed that *A. kamchatica* subsp. *kawasakiana* have extinct or been in endanger in the most reported habitats as shown in a series of checklists of endangered species. In the Red Data Book 2000, Environmental Agency of Japan 2000 it was designated as Vulnerable (VU). It is considered that it had been extinct by 2005 in Shizuoka, Aichi, Osaka, Hyogo and Ehime prefectures (Yamamoto 1978; Ohota et al. 1983; Sugimoto 1984; Environment Agency of Japan 2000; Osaka Prefecture 2000; Aichi Prefecture 2001). In our investigation, Toyama, Mie and Kochi Prefectures, we can locate only single population for each prefecture. The sizes of these populations were small, and it is obvious that immediate conservation of these populations is required. Extant large populations occur on the shore of the Biwa Lake in Shiga

Prefecture.

Even in the localities where the *A. kamchatica* subsp. *kawasakiana* is abundant, the plants occur in restricted habitats. It is likely that there are two conditions for the occurrence of the taxon. First, it requires periodic disturbances during summer that remove competitive perennials. The occurrence of the subspecies always observed in open habitats. Second, the habitats should not be submerged during growth periods of the plants. It is likely that the rises of water levels during summer storms have served as the disturbance agent to fulfill the first requirements. However, because of the second requirement, the plants do not occur in immediate neighbors of water. Therefore, *A. kamchatica* subsp. *kawasakiana* often occurs in narrow belts along boundaries between perennial vegetation and sandy shore.

Other critical aspect on the maintenance of *A. kamchatica* subsp. *kawasakiana* populations is the life cycle as strict annual that requires re-establishment of populations from seeds every year. It is likely that low level of seed dispersion restricts establishments of new populations in distant areas from the extant populations. Then, the above mentioned requirements should be fulfilled repeatedly in the same places. Considering these life history characteristics, habitat conservation from human activities is likely to the only methods to avoid subspecies' extinction.

The results of breeding system experiments indicated that *A. kamchatica* subsp. *kawasakiana* is a self-compatible, non-apomictic species that is able to produce seeds through auto-pollination. High fruit set in the emasculation + hand-pollination + bagging treatment confirmed that our emasculation and bagging treatments did not

interfere with fruit development. Zero fruit set in the emasculation + bagging treatments and high fruit sets in the emasculation + hand-pollination + bagging treatment strongly indicated that the flowers required fertilizations by pollen gains to develop fruits. High fruit set in the bagging treatment suggested that the flowers are self-compatible and have the mechanism for auto-pollination. The results were more or less similar across the three sites that were chosen to cover the geographical range of the subspecies.

Considering the life history of *A. kamchatica* subsp. *kawasakiana* as an annual, increased reproductive assurance through auto-pollination should be critical to produce seeds even when pollinator densities are low, conditions that were observed in the two northern populations in our study. The subspecies occur in sandy sea/lake shores that are exposed to direct sun light and the ground surface temperature often reaches beyond 50°C in daytime during summer. This extremely high summer temperature does not allow plants to maintain perennial rosettes and only seeds can persist summer. Our results do not necessarily indicate that outcrossing is absent or minor in the natural populations. Indeed, at the most southern population, flowers were often visited by the Colleopteran pollinator and outcrossing might be frequent under conditions with high pollinator densities.

Shimizu et al. (2005) reported that *A. kamchatica* subsp. *kawasakiana* has an allotetraploid ($2n = 4x = 32$) origin between two diploid taxa ($2n = 16$), *A. lyrata* and *A. halleri* subsp. *gemmifera.* Both parental species are known to be self-incompatible (Jonsell et al. 1995; Schierup 1998; Mable et al. 2004). These facts and our results indicate that self-incompatibility broke down during the evolutionary process of *A.*

kamchatica subsp. *kawasakiana*. Besides *A. kamchatica* subsp. *kawasakiana*, *A. suecica* (Fr.) Norrl. ex O.E.Schulz is a self-compatible allotetraploid ($2n$ = 26) between *A. thaliana* ($2n$ = 10) and *A. arenosa* (L.) Lawalree ($2n$ =16) (Säll et al. 2003). Tetraploid *A. arenosa* ($2n$ = 32), a putative autotetraploid, is reported to be self-incompatible (Säll et al. 2004). Tetraploid populations of *A. lyrata* subsp. *petraea* (L.) O'Kane & Al-Shehbaz from Austria were strongly self-incompatible but their allo/autopolyploid origins are unknown (Mable et al. 2004). Mable et al. (2005) found that North American populations of *A. lyrata* subsp. *lyrata* included self-compatible individuals. In a recent review in which an association between ploidy levels and self-compatibilities were examined using a large database, Mable (2004) found that polyploidy did not necessarily associate with self-compatibility. It obviously requires more information on breeding systems on populations of *Arabidopsis* species before we correlate polyploidy and loss of self-incompatibility. It is not likely that the life cycle as an annual of the study species is the result of polyploidzation although the two parental diploid species are perennials Another tetraploid subspecies, *A. kamchatica* ssp. *kamchatica* have perennial life cycles, and the subspecies share parental taxa with the studied subspecies (Shimizu et al. 2005).

An interesting question to be asked in future studies is whether the break down of self-incompatibility in *A. kamchatica* subsp. *kawasakiana* occurred as a direct result of allopolyploidization or whether it occurred along with the evolution of the life cycle as an annual.

Chapter 3

Geographic variation of vernalization response in flowering time

Abstract

Geographic variations in the climatic factor along latitudinal gradients often cause differentiation in flowering response to the environmental cues. *Arabidopsis kamchatica* subsp. *kawasakiana* is a winter annual in which flowering is promoted by exposure to cold temperatures (vernalization). To examine whether variation in vernalization response exist among climatic gradients, I conducted growth experiments using four populations. Seeds were collected from natural populations and plants were grown under the controlled conditions in which duration of vernalization treatments were altered. The plant were exposed to 0, 2, 4, 8 week of vernalization and observed day to bolting in a growth chamber. Vernalization accelerated flowering plant from most of the populations and longer cold periods had stronger effects in promoting flowering. We found significance variation in the flowering response along latitude gradients, and northern populations were more sensitive to vernalization, i.e, flowering is promoted even in short cold treatments, than southern populations. Climatic factors that have been selected different flowering responses were assessed.

Introduction

Control of reproductive timing to produce offspring under suitable environmental conditions is a central challenge for organisms, and this challenge is especially

important for semelparous organisms, including annual plants, that reproductive only once in their life cycle. It is known that plants often determine the timing of reproduction by responding to seasonal environmental cues such as, temperature and photoperiod (Simpson and Dean 2002).

The relationship between the environmental cues and the predicted seasonal environment varies with latitude and climate. Consequently, the optimal response to the seasonal cues is likely to vary geographically, leading to geographic differentiation in flowering response. For example, since plants in southern latitudes experience milder winters and earlier spring, they may evolve increased vernalization sensitivity - that is, a more rapid acceleration of flowering time due to a given duration of vernalization exposure (Boudry et al. 2002). In contrast, because winter temperatures are somewhat variable, a "slow" vernalization response may be favored to avoid an occasional warm period during winter (Shindo et al. 2006). In other hypothesis, long cold winters is considered to lead to very limited growth through most of the winter months, necessitating rapid vernalization response once winter has passed (Shindo et al. 2006).

In this chapter, I studied geographic differentiation of vernalization-response in flowering time between natural populations of *Arabidopsis kamchatica* subsp. *kawasakiana*. As it was shown in the previous chapter (Chapter 2), *A. kamchatica* subsp. *kawasakiana* is endemic to Japan and distributes sporadically Hokuriku, Kinki, and Tokai district in western Honshu and Shikoku. These local populations of *A. kamchatica* subsp. *kawasakiana* are likely to be maintained for long enough for local adaptation to shape a geographic pattern in flowering time responses. The distribution ranges from

the Japan Sea side to the Pacific Ocean side. The climate in the Japan Sea side of Honshu is characterized by heavy snowfall during winter, whereas the Pacific side is characterized by small winter precipitation. Therefore, I expected that this contrasting climatic conditions within relatively short geographic ranges resulted in differentiation of flowering response across populations.

I collected seeds from a series of natural populations of *A. kamchatica* subsp. *kawasakiana* along a latitudinal gradient and conducted growth experiments to detect a latitudinal cline in the sensitivity of flowering response to vernalization. I predicted that individuals collected from the lower latitude (south and Pacific Ocean side) require longer vernalization cues to avoid flowering during winter. I also predicted that individuals from the higher latitudes (north and Japan Sea side) do not necessarily require long vernalization, because flowering in the natural habitats is expected to be suppressed by external conditions, such as snow cover and consistent low temperature, during winter.

Material and Methods

Seeds were collected at four natural populations of *A. kamchatica* subsp. *kawasakiana*, i.e. Nakashohama (NSH), Ohmimaiko (OMK), Hikone (HKN) and Fukiiura (FIU) (Table 3-1). The first three populations locate on the shore of the Lake Biwa and the fourth population locates on the shore of Ise Bay. For each population, we collected seeds from seven individuals that were apart more than 5 m from each other. Seeds were sawn on the rock-wool and were germinated under 25℃/15℃ day/night temperatures

with 12 hours day-length. Seedlings were grown on the rock-wool for 28 days in the 25℃/15℃ conditions. During the growth experiments, nutrient was supplied to plants by liquid fertilizer (Hyponex). Four different lengths of cold treatments were applied on these seedlings, i.e. 0, 2, 4 and 8 weeks of cold treatments at 5℃ in dark. We used seven plants for each treatment for each population (one /maternal plant). After the cold treatments, the seedlings were transplanted into plastic pots (7.5 cm in diameter and 6.5 cm in depth) and were grown under 25℃/15℃ day/night temperatures with 12 hours day-length. When the plants were transplanted, we recorded number of rosette leaves and the maximum rosette diameter. We recorded the date of bolting for each plant, and counted number of days to bolting after transplantation. We judged that the plants started bolting when the flowering stems elongated 5 mm or longer. The pants that remain as rosettes without bolting for 120 days after transplantation were recorded as non-flowering plants, and we used the value of 120 days for these plants when we analyzed the data. We obtained meteorological data from the nearest measurement station (Automated Meteorological Data Acquisition System, AMEDAS, of Meteorological Agency, Japan) from each field site. The data from 1980 to 2007 were used to analyze dependency of the results of growth experiments on local climates.

Results

Generally, longer cold treatments resulted in earlier flowering after cold treatments (Fig. 3-1). There were large difference in the ratio of plants that have reached bolting during experiments (Table 3-2), and significant variation in the day to bolting was

detected both populations and treatments in the ANOVA (Table 3-3). NSH and OMK showed flowered earlier compared with HKN and FIU both in terms of ratio of flowering plants and days to flowering. The post hoc test by Fisher's PLSD distinguished the flowering response to the low temperature between the two groups (Table 3-4). For NSH and OMK, almost all individuals flowered within 120 days when they applied cold treatments for 2 weeks or longer. The plants bolted 30-40 days earlier with cold treatments compared the plants under the treatment without chilling. For HKN and FIU, fewer plants bolted under shorter cold treatments (Fig.3-1). The climatic factors (precipitation and temperature) of the study sites during the growing season (from November to March), were summerized in Table3-5. HKN and FIU had tendency to have smaller amount of precipitations during winter compared with NSH and OMK (Table 3-5). The number of warm winter days (defined as days with the >5℃daily minimum temperature) were equivalent across all field sites (Table 3-6).

Discussion

Significant variation of flowering responses to different vernalization periods were detected among *A. kamchatica* subsp. *kawasakiana* populations. Plants from the populations at lower latitude required longer vernalization periods for flowering. The contrasting climatic differences between the Japan Sea side (higher latitude) and the Pacific sides (lower latitude) of the Honshu Island may explain this. Japan sea side is characterized by large precipitation during winter and ground surface is covered by snow for relatively longer period. This snow cover is expected to provide wet and mild

winter temperature near to more or less zero degree. In contrast, the Pacific side is characterized small winter precipitation, and ground surface is exposed to large temperature fluctuations. Soil temperature can be warm enough to allow plants for growth or be freezing cold to below zero degree. The possible adaptive explanations of the observed pattern are that the longer vernalization-requirements functions to avoid flowering during winter in lower latitude (the Pacific side). From the same geographic region, population differentiation in flowering response has also been reported in the other annual Brassicaceae, *Cardamine flexuosa* With., the results were similar with the pattern observed in this study, i.e. plants from the Pacific side require stronger environmental cues to flower compared with those from the Japan Sea side (Kudoh et al. 1995, 1996). To test the above mentioned hypothesis, reciprocal transplant experiments at the field sites are required.

In *A. thaliana*, significant quantitative genetic variation in vernalization responses to flowering has been reported (Karlsson et al. 1993; Nordborg and Bergelson 1999; Shindo et al. 2005). Stinchcombe et al. (2005) reported that southern accessions were more sensitive to vernalization than northern accessions in *A. thaliana*. The pattern was reverse with what we found in this study. We detected a latitudinal cline of flowering response in *A. kamchatica* subsp. *kawasakiana* within a relatively small geographic region, but the reported pattern in *A. thaliana* were detected from much larger geographic ranges. Even in *A. thaliana*, at a local scale across Sweden, southern accessions require longer periods of vernalization for flowering than northern accessions (Shindo et al. 2006). These results suggest that climatic factors of each

location are likely to be critical in selection on the flowering response, but such factors may not vary simply along latitude. In *A. thaliana*, relationship between the expression level of *FLC* and vernalization requirements for flowering have been reported. Pleiotropic effects of *FLC* give another alternative hypothesis to explain the observed pattern. McKay et al. (2003) reported that positive genetic correlation between *FLC* expression level and water use efficiency. If this genetic correlation is shared by related group of plants, selection against low water use efficiency may prefer plants with high *FLC* levels in the Pacific side with dry winter. In this study, climates of the late flowering populations, HKN and OMK, were characterized by small winter precipitation during winter. This pattern did not contradict with the two above mentioned explanations, i.e. avoidance of winter flowering in lower latitude and the pleiotropy with the water use efficiency of *FL*. In any chase, knowledge on genetic mechanism that causes natural variation of vernalization response of *A.kamchatica* subsp. *kawasakiana* is required to synthesize testable hypothesis in further laboratory and field experimental studies.

Chapter 4

Molecular analysis on *AkwFLC*, a putative key factor of vernalization-mediated flowering response

Abstract

I explored the genetic basis of variation in vernalization response of flowering using plants collected from natural populations of allopolyploid *Arabidopsis kamchatica* subsp. *kawasakiana*. In *A. thaliana*, a close relative of *A. kamchatica* subsp. *kawasakiana*, *FLC* is known to be a key factor that control vernalization response. Here, I report the results of characterization of two types of *A. kamchatica* subsp. *kawasakiana FLC* gene (*AkwFLC*) that derived form two parental species. I examined allelic variation of *AkwFLC* across natural populations and found 9 alleles i.e, 6 and 3 alleles of *lyrata* and *gemmifera* types, respectively, in 64 cloned *AkwFLC* sequences from six individuals. The both types shared the amino acid sequences in the MADS box.. Most of strains possessed at least one allele with the 9bp deletion. Total *AkwFLC* transcription level decreased in response to the long cold treatments. Significant variation in the initial transcription levels of *AkwFLC* was detected among populations, but, did not explain flowering time variation under the non-veranalized condition. Although initial levels of *AkwFLC* were higher in NSH, a northern population, NSH respond quickly to the two-week cold treatment and the transcription level became much lower than that of HKN. The transcription levels of *AkwFLC* further decreased after 8-weekcold treatment, but the difference between the two populations were not

grate. These results suggest that sensitivity to the cold rather than the initial level of *AkwFLC* is likely to explain population differentiation of the flowering time response.

Introduction

Control of reproductive timing to produce seeds under a suitable season is especially important for annual plants that reproductive only once in their life cycle. It is known that plants often determine the timing of reproduction by responding to seasonal environmental cues, such as temperature and photoperiod (Simpson and Dean 2002). The relationship between the environmental cues and the predicted seasonal environment varies with latitude and climate. Consequently, the optimal response to the seasonal cues is likely to vary geographically, leading to geographic differentiation in flowering response. Using the techniques of common garden experiments, population differentiation in flowering responses have been detected in many plant species (e.g., Olsson and Ågren, 2002, Kruskopf-Österberg et al., 2002 and Boudry et al., 2002). Molecular bases of population differentiation in flowering-time controls, however, are largely unknown.

In Chapter 3, I detected significant variation in flowering responses to vernalization among populations of *Arabidopsis kamchatica* subsp. *kawasakiana.* The plants from southern populations required longer periods of cold treatments for flowering than those from northern populations (Chapter 3). Molecular and genetic knowledge on the regulatory network of flowering revealed in *Arabidopsis thaliana* (Simpson and Dean 2002; Henderson and Dean 2004; Sung and Amasino 2005;

Mitchell-Olds and Schmitt 2006) allowed me to study mechanistic bases of the observed population differentiation. In this chapter, I investigated molecular mechanisms in geographic variation of flowering response in *A. kamchatica* subsp. *kawasakiana*.

Because of its close relatedness to *A. thaliana*, it is highly producible to consider *A. kamchatica* subsp. *kawasakiana* shares its regulatory mechanisms with the model plant, including number of genes that have been identified in the flowering pathways. Among known flowering-time regulatory genes of *A. thaliana*, I focused on *Flowering Locus C* (*FLC*), a gene coding a MADS Box transcription factor, that is known to be a key regulator of vernalization response (Michaels and Amasino 1999; Sheldon et al. 1999). In *A. thaliana,* high *FLC* expression strongly delay flowering, but this delay in flowering can be fully reversed by prolonged cold treatment (Michaels and Amasino 1999; Sheldon et al. 1999). Vernalization results in epigenetic repression of *FLC* through histone modification that is stable after the cold condition and is maintained during successive spring growth (Sung and Amasino 2006). Repression of flowering by *FLC* and epigenetic silencing of *FLC* in response to prolonged cold temperature are considered to be the mechanism for *A. thaliana* to sense winter and to ensure that flowering occurs after winter (Sung and Amasino 2006).

Aims of the study presented in this chapter are to study the following points;.

(1) Identification of the homologue of *FLC* in *A. kamchatica* subsp. *kawasakiana* (I refer the homologue as *AkwFLC,* hereafter). Because the subspecies is allotetraploid, I cloned *AkwFLC* to identify copies from alternate parental species.

(2) Variation of *AkwFLC* allele and its geographic distribution. I identified sequence variations among different plants from natural populations and developed marker to screen the geographic distribution of a certain type of sequence variation.

(3) Cold response of *AkwFLC*. The amounts of *AkwFLC* RNA were quantified in the growth experiments in that different cold treatments were applied.

(4) Natural variation in the initial transcription level of *AkwFLC*.

(5) Variation in the *AkwFLC* response to cold temperature.

Based on the results, I evaluated what mechanistic bases provide differentiation of vernalization responses among *A. kamchatica* subsp. *kawasakiana* populations.

Material and Methods

Characterization of AkwFLC

We used plants gown from seeds of *Arabidopsis kamchatica* subsp. *kawasakiana* collected at five populations, i.e. Hamakurosaki (HKS), Nakashohama(NSH), Ohmimaiko(OMK), Hikone(HKN), and Fukiiura(FIU) (see Table 2-1 in chapter 2 for the detail information on these localities). Because the study species has the allotetraploid origin, we included strains of putative parental species, i.e., *A. lyrata* collected from North America (locality, Pores Knob, Wilkes Country, North Carolina, USA collected by Dr. K. K. Shimizu at Zurich University) and *A. halleri* subsp. *gemmifera* from a population at the central Hyogo (locality, Omoide-gawa River, Naka-ku, Taka-cho, Taka-gun, Hyogo Pref., Japan, Long. 35°6', Lat. 134°54', Alt. 200 m, collected by Dr. T. Kawagoe at Kobe University). Because *A. kamchatica* subsp.

kawasakiana is expected to have at least two copies of *FLC* homologues of different parental origins, we determined sequence of each copy by cloning. Two individuals from Nakashohama (NSH17 and NSH20), and one from each of other four populations (HKS35, OMK28, HKN45, FIU06) were used for cloning. One strain for each parental diploid species was also cloned (W155 and OID1 for *A. lyrata* and *A.harreli* subsp. *gemmifera*, respectively).

Plants were grown in a growth camber at 25°C under a 12 hours-photoperiod prior to RNA extraction. Total RNA was extracted from these young leaves by using RNeasy Plant Mini Kit (QIAGEN) and treated RNAase-Free DNase set (QIAGEN). Complementary DNA (cDNA) was synthesized from total RNA with High Capacity cDNA Archive Kit (Applied Biosystems). RT-PCR was performed by using Oligo dT20 primer.

By aligning sequences of *FLC* genes of previously reported different plant species (*Arabidopsis thaliana*, *Brassica rapa* subsp *pekinensis*, *B. napus*, *B. rapa* cultivar *samjin*, and *Raphanus sativus*), conserved sequence regions were determined to design primers. Based on the *A. thaliana FLC* (GeneBank Accession No. BK000546) sequence, two primers, i.e., FLC-FU2 (5'-GAAAGAGAAAACGCTTAGTATCTCC-3') and FLC-RD1 (5'-TATGTTTTGGATTTTGATTTCAACC-3'), were designed and were used to amplify the cDNA fragments by PCR from cDNA extracts of study materials. The PCR products were cloned into pGEM®-T Easy vector (Promega) and verified by sequencing. We cloned 8-12 colonies for each strain of *A. kamchatica* subsp. *kawasakiana* (64 strains in total) and sequenced all clones. Sequences of four colonies

were determined for each strain of the parental species. The allotetraploid, *A. kamchatica* subsp. *kawasakiana*, had two sets of sequences each from *A. lyrata* or *A. harreli* subsp. *gemmifera*, and we refer them as lyrata-type and gemmifera-type sequences, respectively, based on the sequence similarities to those determined for the parental species.

An alignment analysis was performed using Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html) against the full-length cDNA sequences for *FLC* obtained from the GenBank. Phylogenetic analyses of *FLC* homologues were performed by the neighbor joining method using the Clustal W version 1.83. Bootstrap values for 1000 time trials were calculated. *AGL20* gene that belongs to the MADS-box gene family is set as an out-group.

Screening of AkwFLC allelic variation

As a result of *AkwFLC* cloning, we detected allelic variations that are likely to affect *FLC* function. One of such allele was found in *A. gemmifera*-origined alleles, a 9bp deletion in the coding region (the *AkwFLC-G3* allele). Interestingly, the identical 9bp deletion was found among *A. lyrata*-originated alleles (the *AkwFLC-L5* allele). Because two 9-bp deletion alleles were found only in the population to which NSH and OMK comparatively flower by a short vernalization, the possibility for these deletion alleles to have brought the differentiation to the flowering response is thought. Therefore, it examined it by using specific primer sets for 9-bp deletion alleles whether to cause bias in the regional distribution of these 9-bp deletion alleles. I designed primers to

distinguish presence and absence of the two alleles with the deletion and screened the distribution of the deletion across natural populations. A forward primer that is specifically complement with the sequence with the 9-bp deletion, *FLCkw-9bdl-Fw* (5'-GCTGATGATCTTAAAGCCTTGTCA-3') and reverse primers that are specific to each of *AkwFLC-G3*, *FLC-kwG-4e1-Rv* (5'-GTGTTCCTCCAATTGAAC AAGAGTA-3') and *AkwFLC-L5*, *FLCkwL-4e1-Rv* (5'-GCTGATGATCTTAAAGCC TTGTAC-3') were used. These primers successfully distinguished the presence/absence of AkwFLC-G3 and AkwFLC-L5. RNA was extracted from 30 strains (5 populations x 6 individuals) of *A. kamchatica* subsp. *kawasakiana*, and c-DNA samples were prepared. We firstly amplified full-length *AkwFLC* and then used them as templates to screen allelic variation.

Vernalization response of AkwFLC

Responses of *AkwFLC* to different length of cold treatments were examined.. A single strain (NSH20) from the Nakashohama population (NSH) was used in this experiment. The seeds were germinated at 25℃/15℃ day/night temperatures with 12 hrs day-length. After three days of the germination, five treatments were applied and then cDNA samples were prepared for the measurement of *FLC* levels. The treatments were 5℃ cold treatment for (1) one, (2) 14 and (3) 28 days, and as controls, (4) one-day cold treatment followed by 13-days incubation at 25℃/15℃ with 12 hrs day-length and (5) one-day cold treatment followed by 27 days. After the treatments, RNA was extracted from each seedling.

Transcription levels of *AkwFLC* in the obtained samples were quantified by real-time PCR. Primer for amplification of PCR products were designed using conserved region of lyrata-type *AkwFLC-L1* and gemmifera-type *AkwFLC-G1* sequences using from Primer Express 2.0 software (Applied Biosystems). Therefore, we putatively considered that the total amounts of *AkwFLC* from both parental origins were quantified using the primers. The primers were FLCkwcomF3e1 (5'-TGGTTCA CACCATGAGCTACTTG-3') and FLCkwcomR4e1 (5'-ACATTATTGACATTTGA TCCCACAA-3'). Primers of UBQ10 (UBQ10Fw-RT: 5'-CACACTCCACTTGGTC TTGCGT-3' and UBQ10Rv-RT: 5'-TGGTCTTTCCGGTGAGAGTCTTCA-3') designed for *Arabidopsis thaliana* were used to quantify one of ubiquitin genes (UBQ10) as an internal control to normalize *AkwFLC* expression. Real-time PCR was performed with ABI PRISM 7300 Sequence Detection System (SDS) (Applied Biosystems, USA) using SYBR Green (Applied Biosystems) to monitor double-stranded DNA synthesis. Each reaction contained 10 μL SYBR Green master mix reagent (Applied Biosystems), cDNA, 500 nM of gene specific primer in a final volume of 20 μL. The optimum primer concentration was checked by template amplifications in the range 50-700 nM for each primer. PCR amplifications were performed using the following thermal profile: 95°C for 10 minutes (hot start), 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension). Data were analyzed using the SDS 1.3 software (Applied Biosystems). To check the specificity of annealing of the primers, dissociation kinetics was performed at the end of each real-time PCR. We applied the standard curve method for relative quantification.

Variation in the Initial Transcriptional Level of AkwFLC

To examine whether the initial levels of *AkwFLC* transcription explain variation in flowering response among populations, *AkwFLC* transcription levels at the seedling stage was quantified for strains from different populations. We used 30 strains from five natural populations (6 for each of HKS, NSH, OMK, HKN and FIU). Seeds were sown on the moistened 15 g crystal sand in petri dishes placed in 25°C /15°C with 12 hrs photoperiod. After two weeks of germination, seedlings were used for RNA extraction. Three replicates per strain were examined. Total RNA was extracted from each seedling by using RNeasy Plant Mini Kit (QIAGEN) and was treated RNAase-Free DNase set (QIAGEN). Complementary DNA was synthesized from total RNA with High Capacity cDNA Archive Kit (Applied Biosystems). RT-PCR was performed by using Oligo dT20 primer.

Quantification of transcription level *of AkwFLC* by real-time PCR was performed on these cDNA samples. Primers for amplification of PCR products were designed using conserved region of all AkwFLC sequence (Table 4-1) from Primer Express 2.0 software (Applied Biosystems). The used primer was FLCkwcomF3e1 (5'-TGGTTCACACCATGAGCTACTTG-3') and FLCkwcomR4e1 (5'-ACATTATTGACATTTGATCCCACAA-3'). Primers for UBQ10 from *Arabidopsis* were used as an internal control to be normalized. Real-time PCR was performed with ABI PRISM 7300 Sequence Detection System (SDS) (Applied Biosystems) using SYBR Green (Applied Biosystems) to monitor double-stranded DNA synthesis. Each

reaction contained 10 μL SYBR Green master mix reagent (Applied Biosystems), cDNA, 500 nM of gene specific primer in a final volume of 20 μL. The optimum primer concentration was checked by template amplifications in the range 50-700 nM for each primer. PCR amplifications were performed using the following thermal profile: 95°C for 10 minutes (hot start), 40 cycle of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing/extension). Data were analysed using the SDS 1.3 software (Applied Biosystems). To check the specificity of annealing of the primers, dissociation kinetics was performed at the end of each real-time PCR.

Variation in the AkwFLC responses to the vernalization

To examine whether the response pattern of *AkwFLC* transcription to the vernalization differ between populations with contrasting flowering response, responses of *AkwFLC* transcription levels to different periods of cold treatments were studied. In chapter 3, I showed that northern populations require shorter period of cold treatment for flowering, whereas southern populations required longer cold treatments for flowering. As representatives of northern and southern populations, I used strains from NSH and HKN, respectively. FIU at the seedling stage was quantified for strains from different populations. Seeds from three strains for each population were sown on the moistened 15 g crystal sand in petri dishes placed in 25°C /15°C with 12 hrs photoperiod. After three days of germination, seedlings were transplanted into plastic containers (4 x 4 x 5 cm in L x W x H) that filled by 2:1 (in volume) mixture of vermiculite and culture soil (N0.4g, P1.0g, and K0.6g /1kg soil, Kureha Kagaku, Japan). The containers were

placed in the growth chamber and plants were grown for six weeks at. 25°C / 15°C with 12 hrs day length. After six weeks of pre-incubation, plants were divided into two groups (three individuals per strain per group) with 2 and 8 weeks of cold treatments. We extracted RNA from leaves and quantified transcriptional levels of *AkwFLC*. As initial values, small leaf tissues were collected from all plants after one day of the initiation of cold treatments, and transcriptional levels of *AkwFLC* were quantified. RNA extraction and successive quantification of *AkwFLC* transcription were conducted with the identical procedures described above.

Results

Characterization of AkwFLC Discussion

I found 9 alleles i.e, 6 (*AkwFLC-L1, L2, L3, L4, L5, L6*) and 3 (*AkwFLC-G1, G2, G3*) alleles of *lyrata* and *gemmifera* types, respectively, in 64 cloned *AkwFLC* sequences from six individuals (Fig. 4-1, Table 4-2). Single unique sequence was obtained for each of the two parental species (Fig. 4-1). The results of phylogenetic analyses strongly supported a clade of 6 lyrata-type alleles of *A. kamchatica* subsp. *kawasakiana* with that of *A. lyrata* and a clade of 3 gemmifera-type alleles with *A.halleri* subsp. *gemmifera*. Lyrata-and gemmifera types of sequences differed in six amino acids substitution which mostly located in the K box of *FLC* (Fig.4-2). The both types shared the amino acid sequences in the MADS box. Among the gemmifera-type sequences except for *AkwFLC-L6* that had a 24bp deletion at the boundary region between MADS-box and I box (Fig.4-3). *AkwFLC-G2* had a single amino acid substitution from leucine to lysine

at the no. 140 position. *AkwFLC-G3* and *AkwFLC-L5* shared 3 amino acid (9 bp) deletions in the I-box region (Fig.4-3). *AkwFLC-L2* and *L3* were characterized by the stop codon at 158th and 156th amino acid. *AkwFLC-L4* had a 42 bp deletion that corresponded the entire region of the sixth exon of *A. thaliana* (Fig.4-3).

Screening of AkwFLC allelic variation

The designed primers successfully distinguished the allelic variation between presence and absence of *AkwFLC-G3* and *AkwFLC-L5* (Fig.4-4). Screening of 30 strains (6 strains x 5 populations) showed that both alleles exist in the all populations (Table 4-3) . Most of strains possessed at least one allele with the 9bp deletion and strains without either type of alleles were found one from each HKS, NSH or FIU population (Table 4-3).

Vernalization response of AkwFLC

AkwFLC transcription level was decreased in response to longer cold treatments (Fig. 4-5). The average levels for plants with 14- and 28-days cold treatments were one third and one seventh, respectively, of that in the control plants with one-day cold treatment (Fig. 4-5). In plants that are grown under 25℃/15℃ conditions after one-day cold treatments, transcription levels of *AkwFLC* increased (Fig. 4-5).

Variation in the initial transcription level of AkwFLC

Significant variation in the initial transcription levels of *AkwFLC* was detected among

populations in the ANOVA (p = 0.0002, Fig.4-6). There was a tendency that early flowering population without vernalization in the growth experiments (chapter 3) had higher initial transcription levels of *AkwFLC*. Spearman's rank correlation coefficient (r=-0.886), however, was not significantly different from zero.

Variation in the AkwFLC responses to the vernalization

Although initial levels (one-day cold) of *AkwFLC* were higher in NSH (41.0) than in HKN (30.8), NSH respond quickly and transcription level of NSH (7.3) became much lower than that of HKN (22.2, Fig. 4-7). The transcription levels of *AkwFLC A* further decreased after 8-weekcold treatment, and the difference between the two populations were not grate (1.1 and 2.1 for NSH and NKH, respectively, Fig. 4-7).

Discussion

The results indicate that *A.kamchatica* subsp. *kawasakiana* has two types of *FLC* −like genes that are presumably originated from two putative parental diploid species. The homology of MADS-box sequences with that of *A. thaliana* was high, and the genes responded to vernalization at least in the transcriptional level. Therefore, I judged that the obtained sequences are homologues of *FLC*. Furthermore, it turned out that the both types of *AkwFLC* were transcribed, although I found several alleles with deletions. It has been reported that in polyploidy with an old origin one of the duplicated genes often becomes seudo-genes, in which function of one of the gene has been lost. It has been considered that the presence of the other functional gene can prevent the fitness loss,

and therefore, natural selection can maintain the function of either of duplicated genes. The results may suggest the origin of allopolyploidy of the study species is relatively new. I detected three gemmifera-type and six lyrata-type alleles that were transcribed in the samples of 30 strains collected from five populations. One of the largest deletions was 9bp, and I determined geographic variation of this deletion. However, the alleles with this deletion were found from all populations. It is not likely that the distribution of this allele explain geographic differentiation of flowering responses. To determine whether or not these alleles are functional or not, we need to construct transgenic plants.

The sensitivity of *AkwFLC* transcription to the cold treatment is likely to explain variation in the flowering response between natural populations of *A. kamchatica* subsp. *kawasakiana*. In the plants from one of the northern populations, in which flowering can be induced by relatively short vernalization, the *AkwFLC* transcription level decreased quickly than that was observed for the plants from the southern populations. I also quantified the initial levels of *AkwFLC* to test whether it explain variation of flowering time responses. I expected that higher initial expression in the plants from southern populations. The results, however, showed the rather reverse tendency, and the initial levels of *AkwFLC* did not explain variation in the flowering time response.

Several studies have reported mechanistic background of natural variation in flowering time. In allopolyploid *Brassica napus* L., late flowering and responsiveness to vernalization correlated with the level of *BnFLC* mRNA expression (Tadege et al. 2001). In *Brassca*, number of alleles possessed by polyploidy are suggested to enlarge

variation of flowering responses (Osborn 2004). In *Capsella bursa-pastoris* (*Brassicacease*), using *Arabidopsis* microarrays, the gibberellin and photoperiodic flowering pathways were significantly different between early- and late-flowering ecotypes, but not for *FLC* expression (Slotte et al. 20007). In *A. thaliana*, it has been reported that allelic variation in *FLC* and epistatic interaction with other genes that regulate *FLC* (e.g. *FRI*) cause, at least in part, natural variation of flowering responses. Epistatic interaction with *FRI* explained variation in flowering response of *A. thaliana* at a large geographic scale covering Eurasian and North Africa, whereas a smaller scale of geographic variation in Sweden was explained by stability of suppressed state of *FLC* (Shindo et al. 2006). The genetic basis of the variation in the vernalization response way differs depending on the species and geographic scales.

Chapter 5

General discussion

It is suggested that geographic cline in flowering response of *A. kamchatica* subsp. *kawasakiana* is attributable to the speed of suppression in *AkwFLC*, a homologue of flowering repressor FLC in *A. thaliana*, in response to exposure to cold temperature (Chapter 4). Geographic clines in flowering responses have been reported from other Brassicaceae. In the same geographic region, highly genetic variation in flowering response of *Cardamine flexuosa* has been reported. They found similar type of geographic cline with this study, i.e. later flowering genotypes in the southern populations (Kudoh et al., 1995, 1996). Stinchocombe et al. (2004) showed, in a common garden experiment, that plants from higher-latitudes flowered later than those from lower latitudes. The geographic cline in flowering time was, however, observed only among individuals with functional *FRI* alleles. The cline disappeared when the analyses were made on individuals with putatively functionless *FRI* allele. The result indicates that *FRI* genotype determines whether natural selection acts and produces geographic cline in flowering time. Furthermore, Caicedo et al. (2004) found that epistasis between *FLC* and *FRI* mediated distribution of *FLC* alleles across natural populations. Among ecotypes with functional *FRI*, frequency of specific *FLC* alleles differed between northern and southern populations.

We found multiple alleles of *AkwFLC*, but it did not explain natural variatioin in flowering time (Chapter 4). Our field investigation presented in Chapter 2 revealed

that *A. kamchatica* subsp. *kawasakiana* exists as small geographically disjunctive populations. Furthermore, we estimated that the study subspecies reproduces predominantly through inbreeding (Chapter 2). The geographic population structure and the breeding system are likely to prevent current gene flow among populations and genetic differentiation is likely to occur among populations. Because many alleles of *AkwFLC*were found across multiple populations, these alleles are likely to have old origins. We need to know whether we can find these alleles in the parental populations. Although the population size judged from growing plants were small, soil seed bank may have served as a reservoir of genetic variation. We found that, in several localities, considerable number of plants occurs solely from soil seed bank (Yamaguchi et al. unpublished). Formation of soil seed bank may explain maintenance of shared allelic variation in *AkwFLC* across multiple populations.

Although it was not possible to quantify *AkwFLC* from different parental origins, in future, we need to study the role of allopolyploidization in producing natural variation in flowering responses.. Recently, effects of polyploidization, or genome duplication, on gene regulation have become studied intensively (Adams and Wendel 2005). *Arabidopsis suecica* is an allopolyploid originated from the hybridization between *A. thaliana* and *A. arenosa*, providing the opportunities to study the consequences of plant pollyploidizations. Wang et al. (2006) studied the expression patterns of *FLC* and *FRI* in the synthetic allopolyploid produced by hybridizing *A. thaliana* and *A. arenosa.* They found that *FRI* with the *A. arenosa* origin regulated *FLC* with the *A. thaliana* origin in the synthetic allopolyploid. The genus *Brassica* also

includes many allopolyploid species. Four *FLC* loci were identified in *B. rapa*, allowing much larger combination of *FLC* alleles within and among *FLC* loci. Schrnaz et al. (2002) showed that there was an association between the number of late-flowering *FLC* alleles and flowering time. In *B. napus*, five orthologues of *FLC* were identified. Although *FLC* is expressed in a whole body including root origin in *A. thaliana*, it is not expressed in root in *B. napus*. Furthermore, each *FLC* copy differed in their relative expressions in different organs. When multiple copies of a gene are produced by polyploidization, some copies may become silenced by methylation within a few generations. It is a challenging issue to clarify how gene duplication affects the evolution of phenotypic plasticity of flowering time during the evolution of the study subspecies and related group of plants..

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Table 2-1. Three study sites used in this study. Latitude, longitude, altitude, area, population size and number of flowering plants are listed.

Site	Latitude (N)	Longitude (E)	Altitude (m)	Area	Population size (number of plants)	Number of flowering plants (2003)
Hamakurosaki	36°45'	137°16'	3	40 x 60 m	300 - 400	147
Nakashohama	35°26'	136°06'	86	10 x 2,000 m	> 10,000	> 1,000
Fukiiura	34°35'	136°38'	5	20 x 40 m	200 - 300	127

Table 2-2. Numbers of tested flowers and matured fruits and fruits set for four different treatments (open, bagging, emasculation, + bagging and emasculation + hand-pollination + bagging) in the three study sites. Results of chi-square tests against the open-pollination treatment (**, $P < 0.01$, and ns, $P > 0.05$) are also listed.

Treatments	Sites	Tested flowers	Matured fruit	Fruits set	χ^2
Open pollination	Hamakurosaki	14	13	0.93	
	Nakashohama	51	50	0.98	
	Fukiiura	9	8	0.89	
Bagging	Hamakurosaki	14	14	1.00	1.04 ns
	Nakashohama	51	51	1.00	1.01 ns
	Fukiiura	9	9	1.00	1.06 ns
Emasculation + bagging	Hamakurosaki	14	0	0.00	24.27 **
	Nakashohama	51	0	0.00	98.08 **
	Fukiiura	9	0	0.00	14.40 **
Emasculation + hand-pollination + bagging	Hamakurosaki	14	12	0.86	0.37 ns
	Nakashohama	51	45	0.88	3.80 ns
	Fukiiura	9	9	1.00	1.06 ns

Table 3-1. Four study sites used in this study. Latitude, longitude, altitude, area, population size and number of flowering plants are listed.

Site	Abbreviation	Latitude (N)	Longitude (E)	Altitude (m)	Area	Population size (number of plants)
Nakashohama	NSH	35°26'	136°06'	86	10 x 2,000 m	> 10,000
Ohmimaiko	OMK	35°13'	135°57'	87	50 x 40 m	100-200
Hikone	HKN	35°14'	136°09'	87	20 x 50 m	200-300
Fukiiura	FIU	34°35'	136°38'	5	20 x 40 m	200 - 300

Table 3-2. Numbers of plants used and non-flowering plants at the end of the experiments. Non-flowering plants represent those that remain as rosettes without bolting for 120 days after transplantation.

Chiling tretment (week)	NSH		OMK		HKN		FIU	
	N	No. of non-flowering	N	No. of non-flowering	N	No. of non-flowering	N	No. of non-flowering
0	7	2	7	0	7	5	7	5
2	5	0	7	0	6	4	6	3
4	7	0	7	0	7	3	6	1
8	7	0	7	0	5	2	6	0

Table 3-3. Results of the two way ANOVA for the days to bolting in *A. kamchatica* subsp. *kawasakiana.*

Source of variance	df	Mean Square	*F*	*P*
Population	3	14316.7	20.10	<0.0001
Treatment	3	13542.6	19.01	<0.0001
Population X Treatment	9	564.6	0.79	7.13
Error	88	712.1		

Table 3-4. Post hoc test for the days to bolting of *A. kamchatica* subsp. *kawasakiana* analyzed by Fisher' PLSD.

Effect	Difference of mean value	*P*
Population		
NSH X OMK	3.901	0.5928
NSH X HKN	44.705	<0.0001
NSH X FIU	33.145	<0.0001
OMK X HKN	48.606	<0.0001
OMK X FIU	37.046	<0.0001
HKN X FIU	−11.560	0.1292
Treatment		
0 week X 2 weeks	30.167	0.0001
0 week x 4 weeks	42.657	<0.0001
0 week X 8 weeks	53.810	<0.0001
2 weeks X 4 weeks	12.491	0.0988
2 weeks X 8 weeks	23.643	0.0026
4 weeks X 8 weeks	11.153	0.1357

Table 3-5. Twenty-eight years (1980-2007) average in monthly precipitation, and monthly mean of daily mean, maximum and minimum temperature for the four nearest meteorological observatories from the study sites.

	Nakashohama	Ohmimaiko	Hikone	Fukiiura
Name o fmeteorologica lobservatory	Imazu	Minamikomatu	Hikone	Obata
Latitude(N)	35° 24'7	35° 14'1	35° 16'5	34° 31'7
Longitude(E)	136° 1'7	135° 57'4	136° 14'6	136° 39'9
Altitude (m)	88	90	87.3	10
November				
Mean of precipitation (mm) ±S. D.	112.6 ±48.8	101.8 ±54.2	85.7 ±46.9	111.6 ±90.6
Mean of temperature (℃) ±S. D.	10.3 ±1.33	11.2 ±1.23	11.4 ±1.19	11.7 ±1.27
Mean of maximum temperature (℃)±S. D.	14.4 ±1.28	15.0 ±1.08	15.3 ±1.17	16.4 ±1.13
Mean of minimum temperature (℃)±S. D.	6.3 ±1.40	7.5 ±1.29	7.5 ±1.29	7.2 ±1.53
December				
Mean of precipitation (mm) ±S. D.	147.3 ±54.9	106.9 ±39.9	91.6 ±36.6	50.7 ±43.9
Mean of temperature (℃) ±S. D.	5.1 ±1.20	6.1 ±1.07	6.2 ±1.12	6.6 ±0.96
Mean of maximum temperature (℃)±S. D.	8.6 ±1.32	9.5 ±1.12	9.7 ±1.16	11.4 ±0.96
Mean of minimum temperature (℃)±S. D.	1.8 ±1.04	2.8 ±0.96	2.8 ±1.06	2.1 ±1.01
January				
Mean of precipitation (mm) ±S. D.	165.8 ±56.0	121.7 ±44.4	109.7 ±36.8	55.1 ±51.0
Mean of temperature (℃) ±S. D.	2.5 ±1.17	3.6 ±1.02	3.6 ±1.07	4.5 ±0.96
Mean of maximum temperature (℃)±S. D.	5.5 ±1.38	6.7 ±1.08	6.8 ±1.14	8.8 ±1.01
Mean of minimum temperature (℃)±S. D.	−0.5 ±1.14	0.6 ±0.89	0.6 ±0.94	0.3 ±0.95
Febrary				
Mean of precipitation (mm) ±S. D.	129.2 ±44.8	128.4 ±54.9	98.1 ±39.5	68.3 ±45.1
Mean of temperature (℃) ±S. D.	2.7 ±1.43	3.6 ±1.30	3.8 ±1.31	4.7 ±1.12
Mean of maximum temperature (℃)±S. D.	5.9 ±1.76	6.9 ±1.45	7.2 ±1.45	9.3 ±1.27
Mean of minimum temperature (℃)±S. D.	−0.6 ±1.34	0.4 ±1.19	0.7 ±1.17	0.4 ±1.14
March				
Mean of precipitation (mm) ±S. D.	132.1 ±36.6	152.6 ±46.5	120.4 ±33.8	126.3 ±45.1
Mean of temperature (℃) ±S. D.	5.9 ±1.18	6.7 ±1.01	6.8 ±1.01	8.0 ±1.06
Mean of maximum temperature (℃)±S. D.	9.9 ±1.36	10.6 ±1.11	10.9 ±1.19	12.6 ±1.22
Mean of minimum temperature (℃)±S. D.	1.9 ±1.17	2.8 ±1.02	3.1 ±0.97	3.2 ±1.17

Table 3-6. Average number of warm winter days (with ≥ 5℃daily minimum temperature) and the maximum record for continuous warm winter days between 1980 to 2007.

	Nakashohama			Ohmimaiko			Hikone			Fukiiura		
	December	January	Febrary	December	January	Febrary	December	January	Febrary	December	January	Febrary
Number of warm winter days ±S.D.	2.9 ±2.5	0.8 ±1.4	0.9 ±1.7	5.6 ±3.7	1.4 ±1.7	1.5 ±2.2	5.9 ±3.8	1.4 ±1.7	1.7 ±2.3	4.6 ±3.0	1.5 ±1.8	2.0 ±2.3
The maximum record for continuous warm winter days	4	3	3	8	3	3	9	3	3	4	4	4

Table 4-1. Sequence of the real time PCR primers region of FLC and its homologues of *A. thaliana, A. halleri* subsp. *gemmifera, A. lyrata* subsp. *lyrata and* nine *A. kamchatica* subsp. *kawasakiana.*

	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
	7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 9 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6
A. thaliana FLC	T G G T T C A C A C **T** A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A **A** A A T G T
A. gemmifera FLC	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
A. lyrata FLC	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-G1	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-G2	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-G3	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L1	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L2	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L3	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L4	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L5	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
Akw FLC-L6	T G G T T C A C A C C A T G A G C T A C T T G A A C T T G T G G A A A G C A A G C T T G T G G G A T C A A A T G T C A A T A A T G T
FLCkwcomF3e1 primer	T G G T T C A C A C C A T G A G C T A C T T G
FLCkwcomR4e1 primer	(positions 312–336) T T G T G G G A T C A A A T G T C A A T A A T G T

Table 4-2. Number of colonies verified by sequencing in *AkwFLC* of *Arabidopsis kamchatica* subsp. *kawasakiana* in 6 study sites.

	HKS35	NSH17	NSH20	OMK28	HKN45	FIU06
AkwFLC-G1	2	5	3	6	4	3
AkwFLC-G2	3					2
AkwFLC-G3			2			
AkwFLC-L1		6	6	2		6
AkwFLC-L2	2					
AkwFLC-L3					2	
AkwFLC-L4	1				6	
AkwFLC-L5				2		
AkwFLC-L6				1		

Table 4-3. Results of screening of 9 bp deletion alleles used each specific primer in six study sites. Asterisk (*) is detected 9bp deletion allele. Hyhyen (-) is not detected 9bp deletion allele.

	Gemmifera type 9 bp deletion	Lyrata type 9 bp deletion
HKS35	*	*
HKS38	*	-
HKS39	*	*
HKS40	-	*
HKS42	-	-
HKS43	*	*
NSH17	-	*
NSH20	*	-
NSH21	-	-
NSH23	-	*
NSH25	*	*
NSH32	*	*
OMK28	-	*
OMK31	-	*
OMK32	-	*
OMK33	*	-
OMK34	*	*
OMK35	-	*
HKN45	-	*
HKN46	*	*
HKN49	*	*
HKN50	*	-
HKN51	*	*
HKN52	*	*
FIU01	*	*
FIU02	*	*
FIU03	*	*
FIU05	*	*
FIU06	-	-
FIU07	*	*

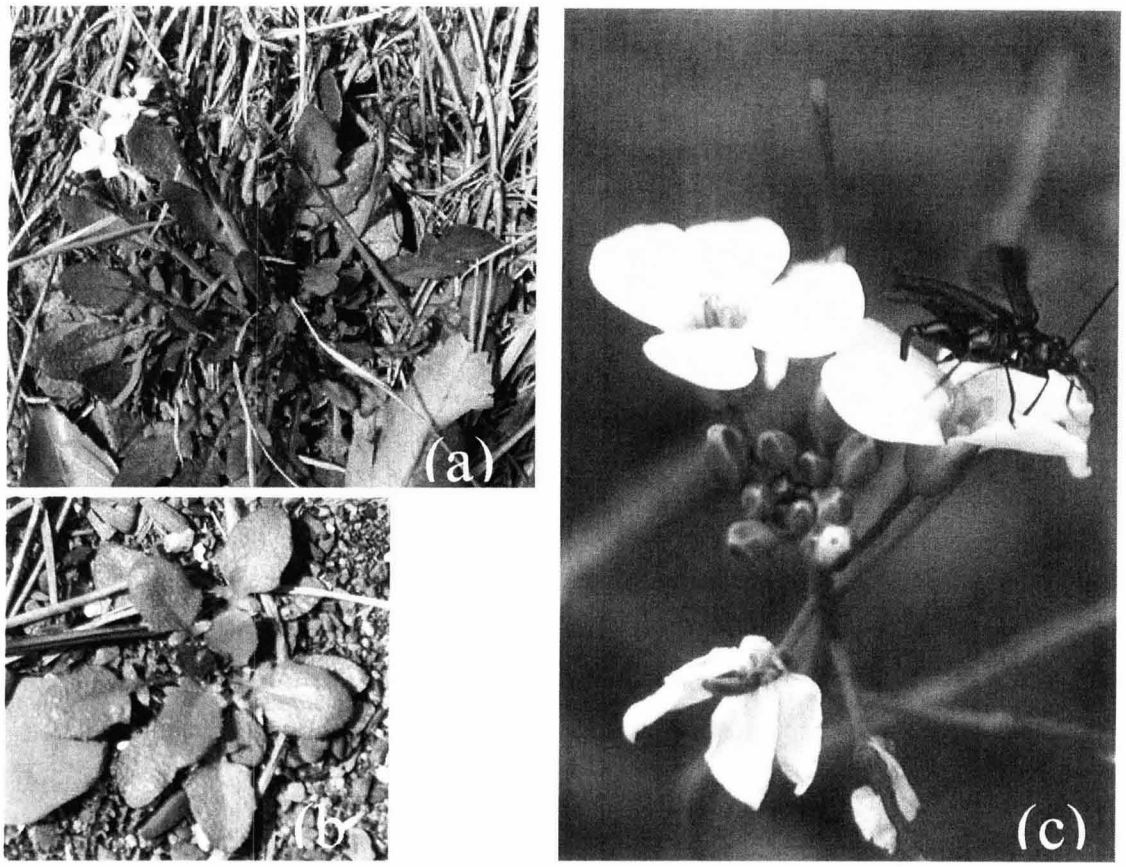

Fig. 2-1 *Arabidopsis kamchatica* subsp. *kawasakiana* in flowering (a), at vegetative stage (b), and a small Coleopteran pollinator, *Oedemeronia lucidicollis* Motschulsky, visiting flowers (c).

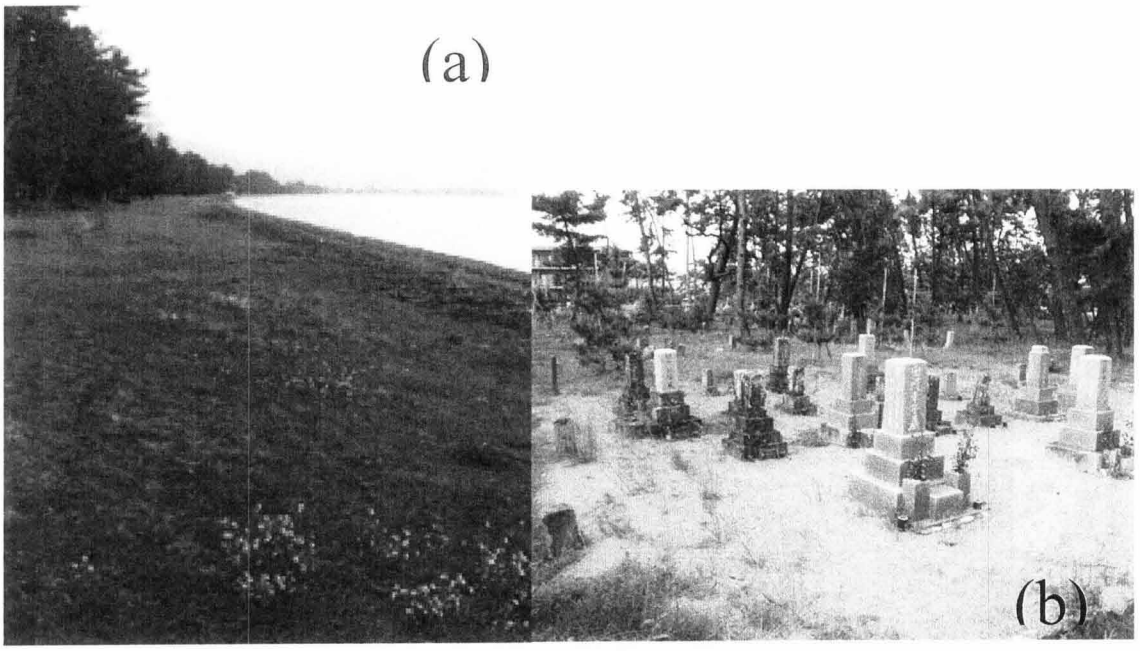

Fig. 2-2 Typical habitats of *Arabidopsis kamchatica* subsp. *kawasakiana* along sandy lake shore at Nakashohama (NSH, a) and a graveyard on sand near to the sea shore at Tanesaki (b).

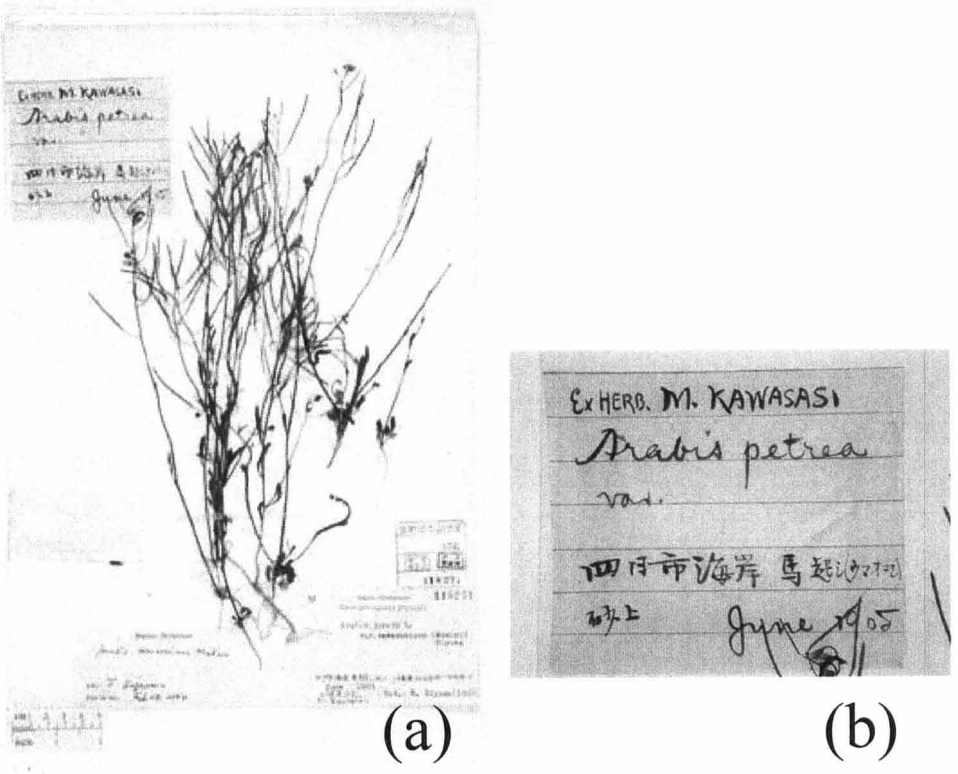

Fig. 2-3 Type specimen *of Arabidopsis kamchatic*a subsp. *kawasakiana* deposited at MAK (a) and the label on the specimen (b).

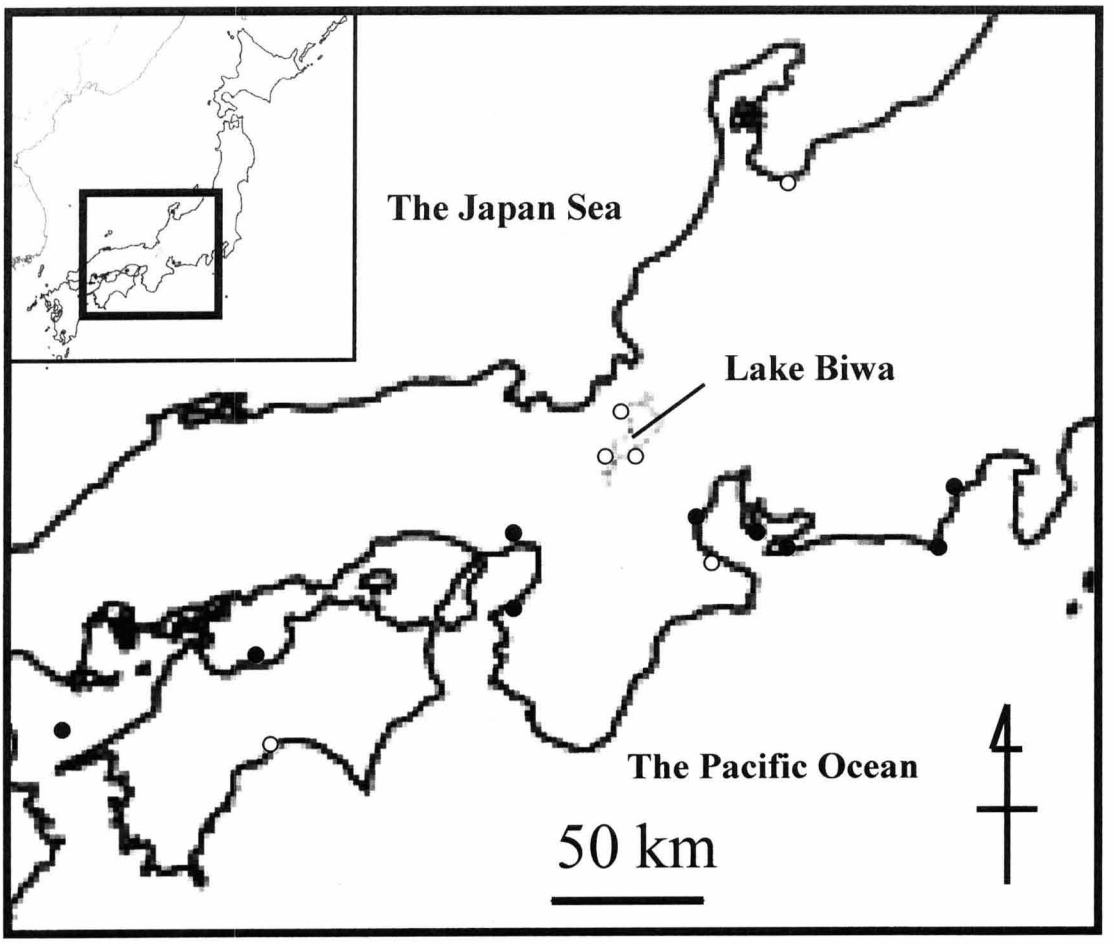

Fig. 2-4 Distribution map of *Arabidopsis kamchatica* subsp. *kawasakiana.* White circles indicate extant populations in which the occurrence of plants was confirmed in this study. Black circles are the extinct localities with records only in literatures.

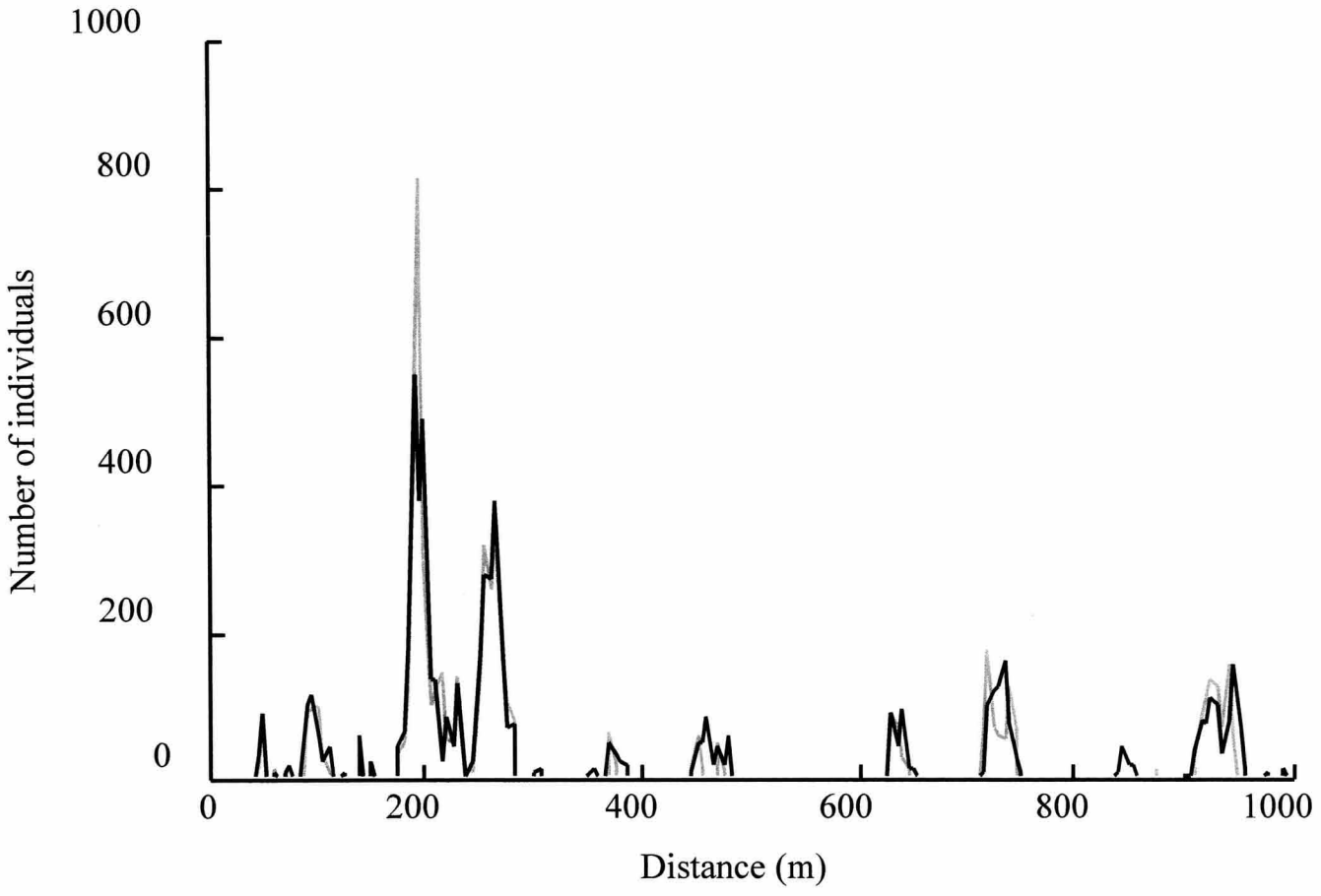

Fig. 2-5 Spatial distribution of *Arabidopsis kamchatica* subsp. *kawasakiana* individuals along a transect (5 x 1000 m) at Nakashohama site in 2002 (Black bar) and 2003 (Gray bar).

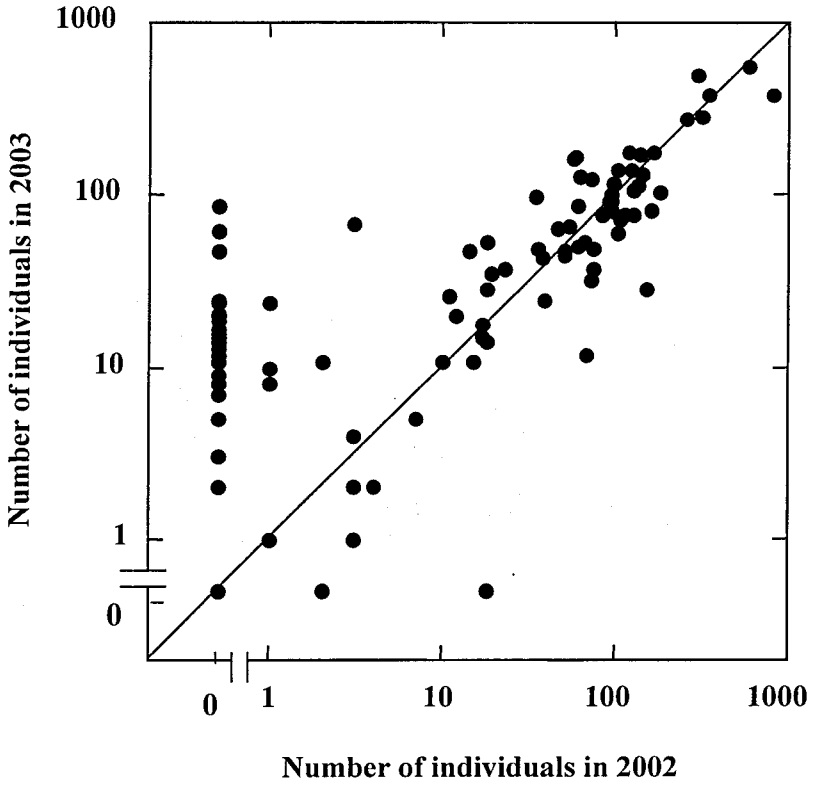

Fig. 2-6 Number of *Arabidopsis kamchatica* subsp. *kawasakiana* individuals within 25 m^2 sub-quadrats in the belt transect at Nakashohama populations in 2002 and 2003. A line indicates that the numbers were identical between the two years if plots are on the line

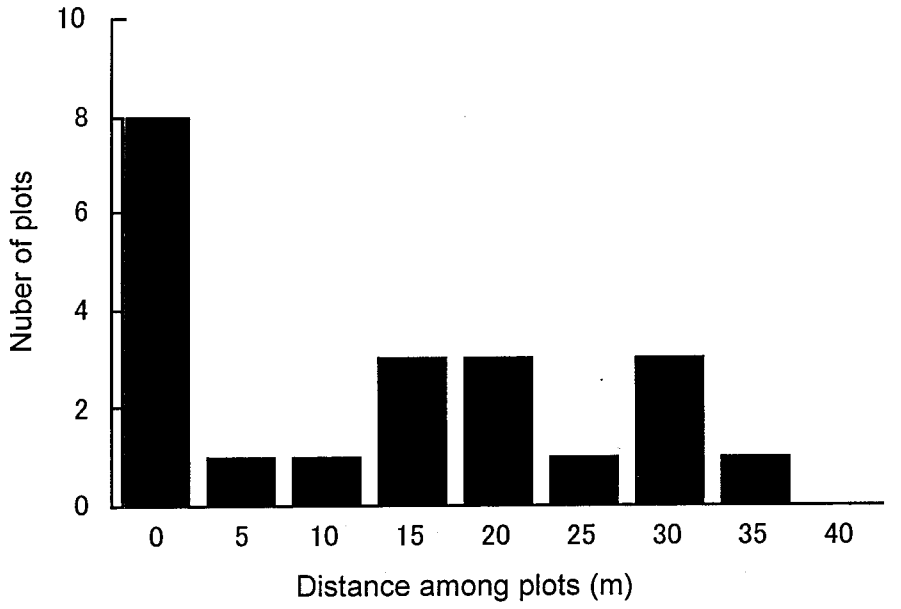

Fig. 2-7 Distances between the quadrats with new introduction of *Arabidopsis kamchatica* subsp *kawasakiana* in 2003 (plants were absent in 2002 and present in 2003) and the nearest quadrats with plants in the previous year (2002).

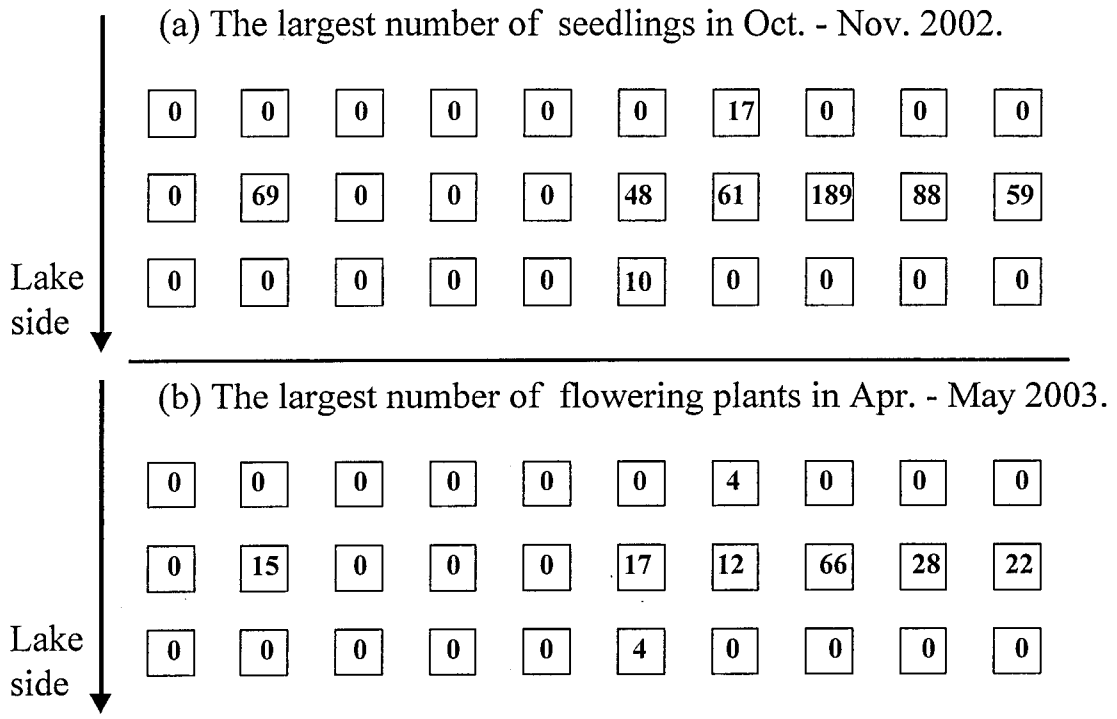

Fig. 2-8 Spatial distribution of *Arabidopsis kamchatica* subsp. *kawasakiana* individuals. The numbers within 1 m x 1 m quadrats arranged in Nakashohama sites are shown. (a) The largest number of individuals during seedling stage (Oct. – Nov., 2002, a) and during flowering season (Apr. - May, 2003, b).

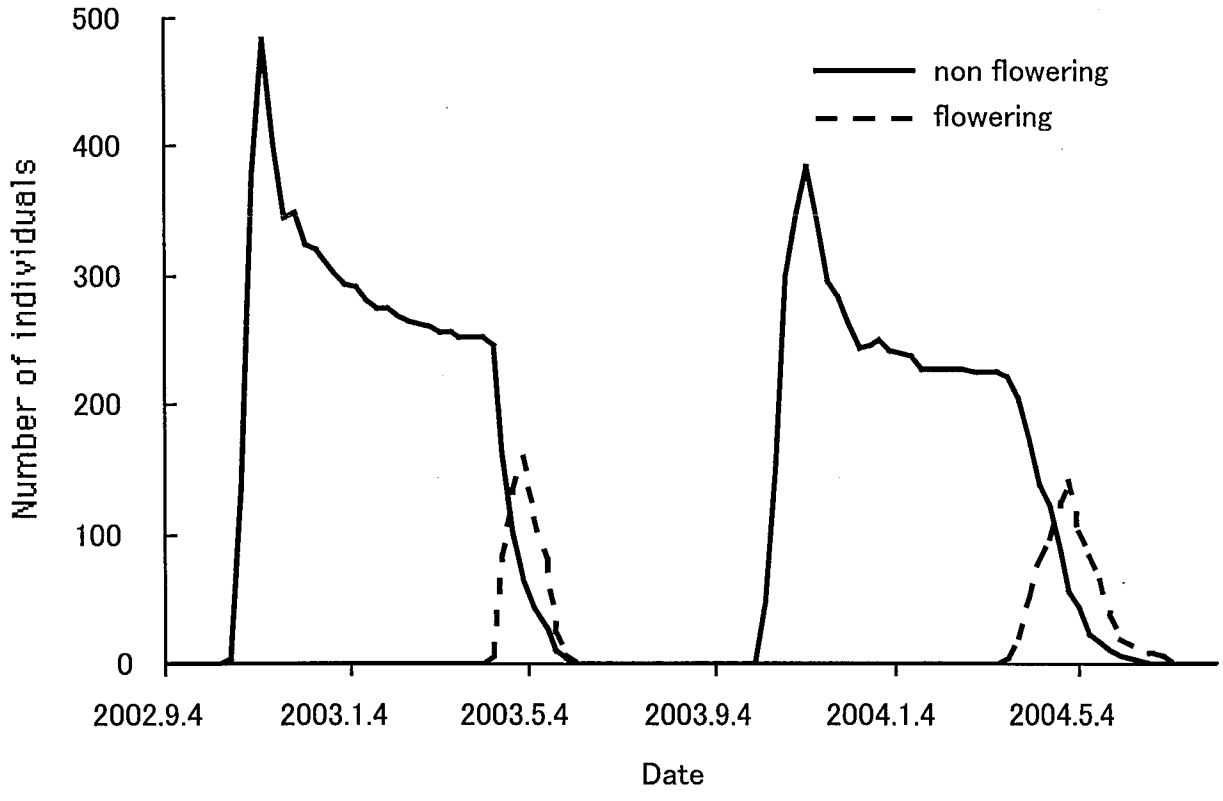

Fig. 2-9 Phenology of *Arabidopsis kamchatica* subsp. *kawasakiana* shown by changes in average number of flowering and non-flowering individuals per 1 m^2 quadrat from Sep. 2002 to Apr. 2004 at Nakashohama (NSH).

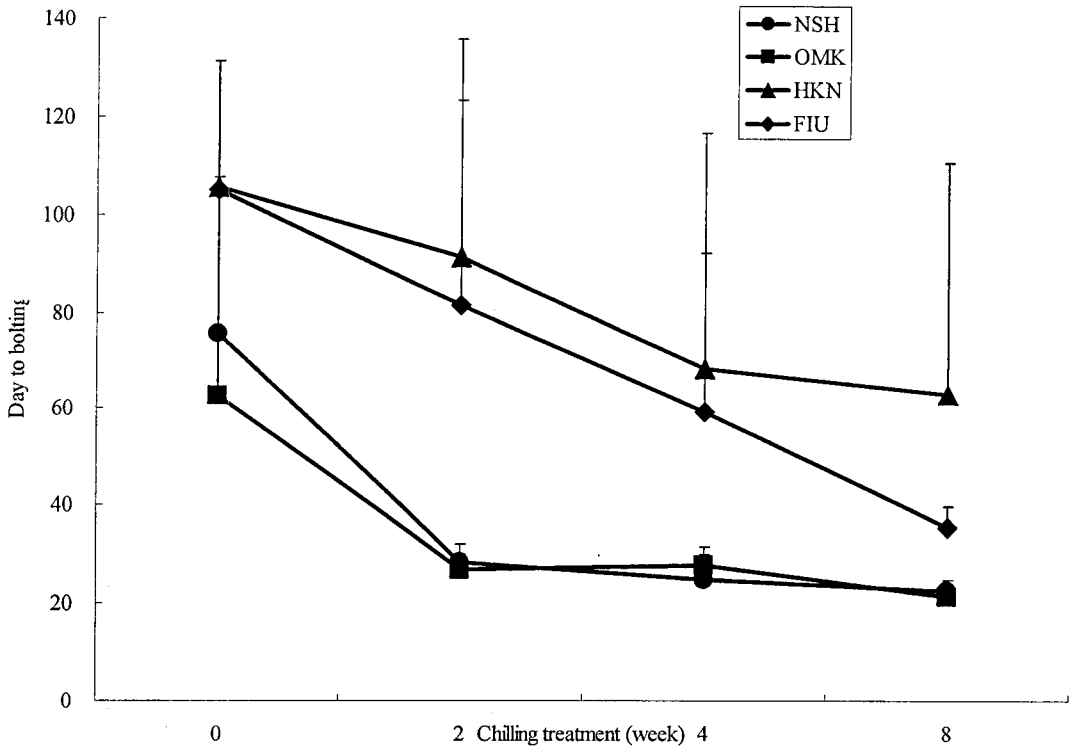

Fig.3-1 Variation in vernalization response of *Arabidopsis kamchatica* subsp. *kawasakiana*. Mean days to bolting under four different length of chilling treatments are plotted for four populations (six plants per population). The plants that remain as rosettes without bolting after 120 days experimental periods were recorded as non-flowering plants, and values of 120 days were used for these plants in calculating averages. The error bars represent standard deviations.

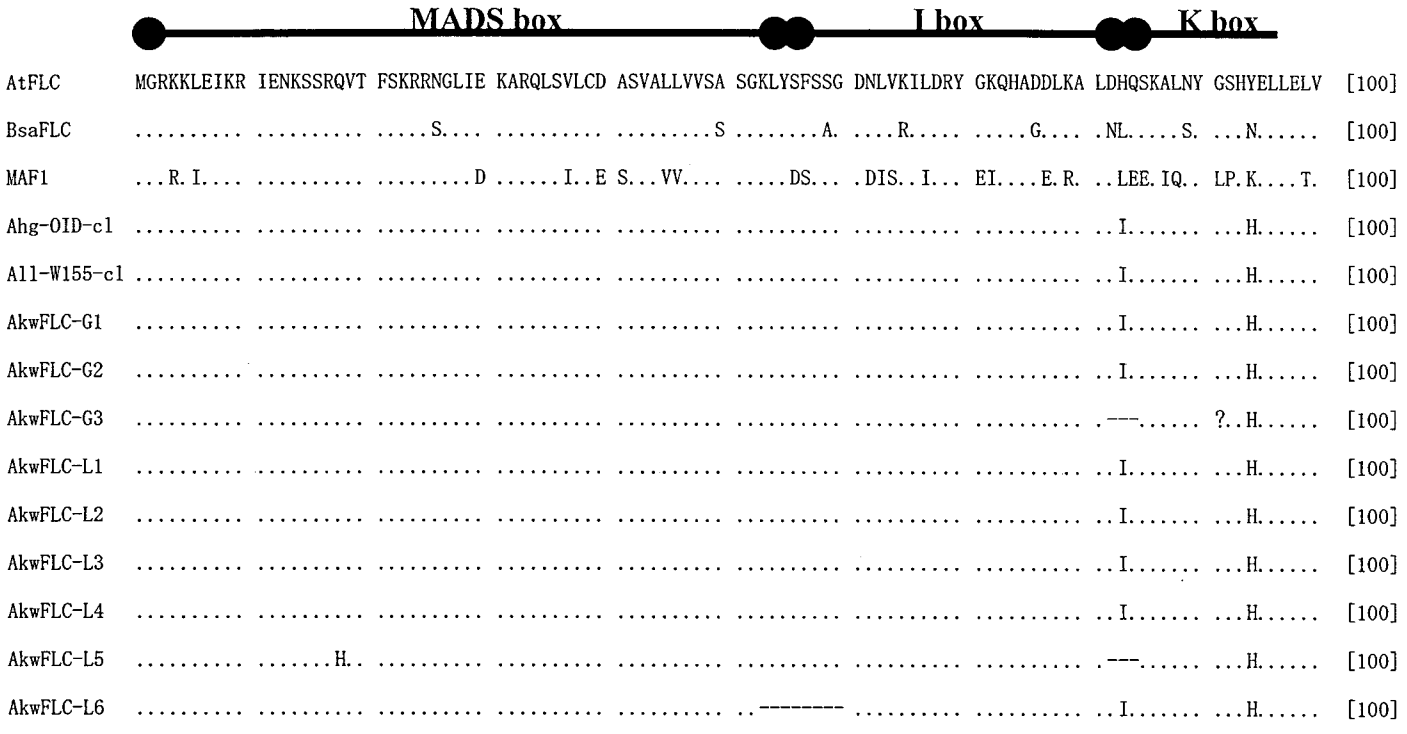

Fig. 4-1 Amino acid sequences of *AkwFLC* (*FLC* homologue in *Arabidopsis kamchatica* subsp. *kawasakiana*). Sequences of *Arabidopdid thaliana FLC, MAF1*, and two putative parental species, *A. lyrata* and *A. halleri* subsp. *gemmifera FLC* homologues are also listed. Two types of *AkwFLC*were designated by L (*A. lyrata* type) or G (*A. halleri* subsp. *gemmifera* type) following hyphens.


```
AtFLC          DSKLVGSNVK NVSIDALVQL EEHLETALSV TRAKKTELML KLVENLKEKE KMLKEENQVL ASQMENNHHV GAEAEMEMSP AGQISDNLPV TLPLLN*  [197]
BsaFLC         .....E...G G..V.T.... .GV..N...L ...R..... ...DS..... .L......A. .G.K.KKNLA ....DNMEMS P.....INLP VTLP.LN* [198]
MAF1           Q...EEP..D ...V.S.IS. ..Q..... S..R.A...M EYI.S..... .L.R..... ....GK.TLL ATDD.RG.F. GSSSGNKI.E .......  [197]
Ahg-OID-cl     E........N ...V.T.... ...F..... .......... .......... .L........ .....K.... .......I.. .......... .......  [197]
All-W155-cl    E........N ...AET.L.. .......... .......... .......... .L........ .....K.... .......I.. .......... .......  [197]
AkwFLC-G1      E........N ...V.T.... ...F..... .......... .......... .L........ .....K.... .......I.. .......... .......  [197]
AkwFLC-G2      E........N ...V.T.... ...F..... .........K .......... .L........ .....K.... .......I.. .......... .......  [197]
AkwFLC-G3      E........N ...V.T.... ...F..... .......... .......... .L........ .....K.... .......I.. .......... .......  [197]
AkwFLC-L1      E........N ...AET.L.. ......T... .......... .......... .L........ T....K.... .......I.. .......... .......  [197]
AkwFLC-L2      E........N ...AET.L.. ......T... .......... .......... .L.....*.. T....K.... .......I.. .......... .......  [197]
AkwFLC-L3      E........N ...AET.L.. ......T... .......... .......... .L...*.... T....K.... .......I.. .......... .......  [197]
AkwFLC-L4      E........N ...AET.L.. ......T... .........- ---------- ---..K.... .......I.. .......... .......  [197]
AkwFLC-L5      E........N ...AET.L.. ......T... .......... .......... .L........ T....K.... .......I.. .......... .......  [197]
AkwFLC-L6      E........N ...AET.L.. ......T... .......... .......... .L........ T....K.... .......I.. .......... .......  [197]
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Fig. 4-1 continue.

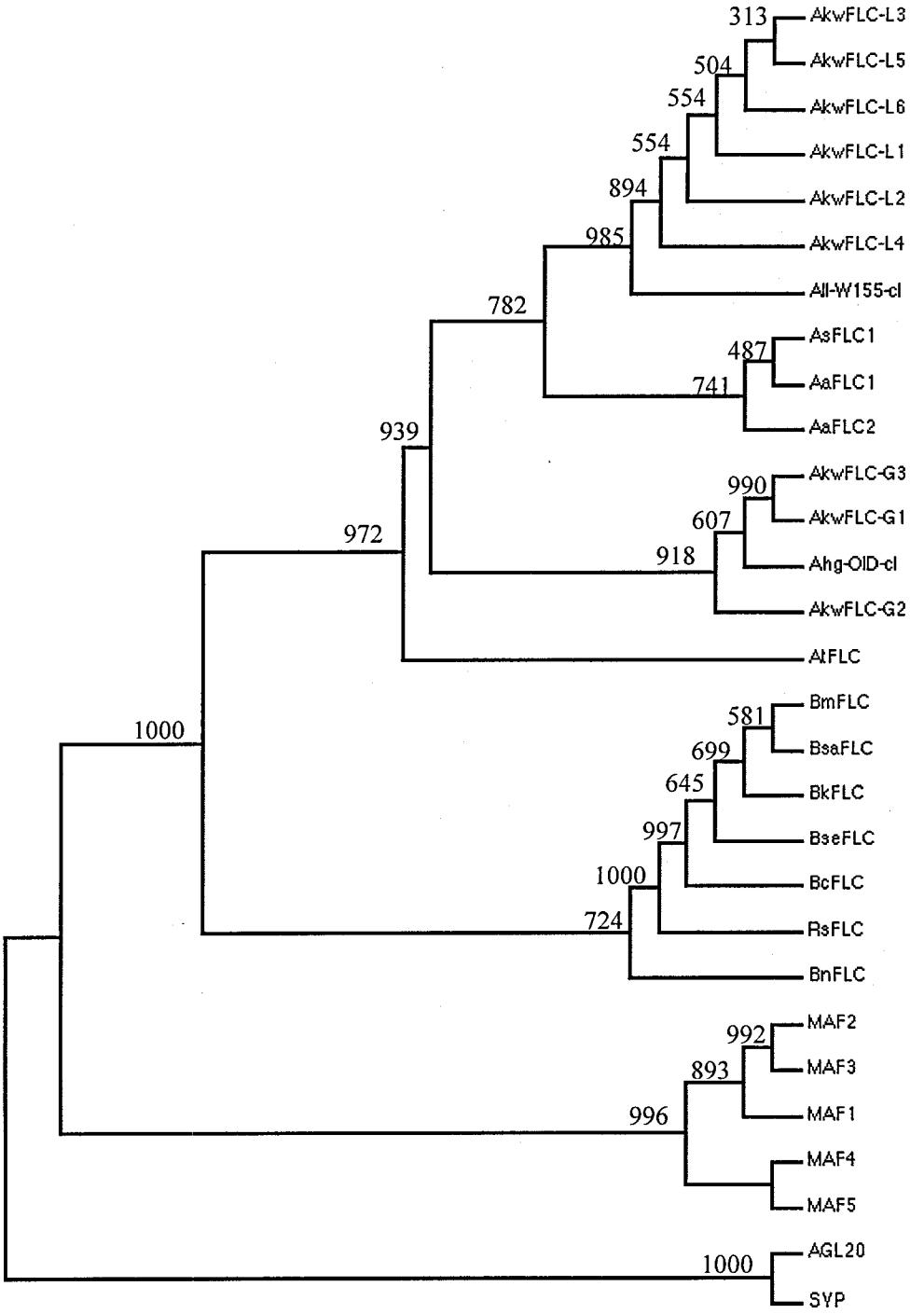

Fig. 4-2 A tree constructed based on nucleotide sequences of *AkwFLC* and other related genes.

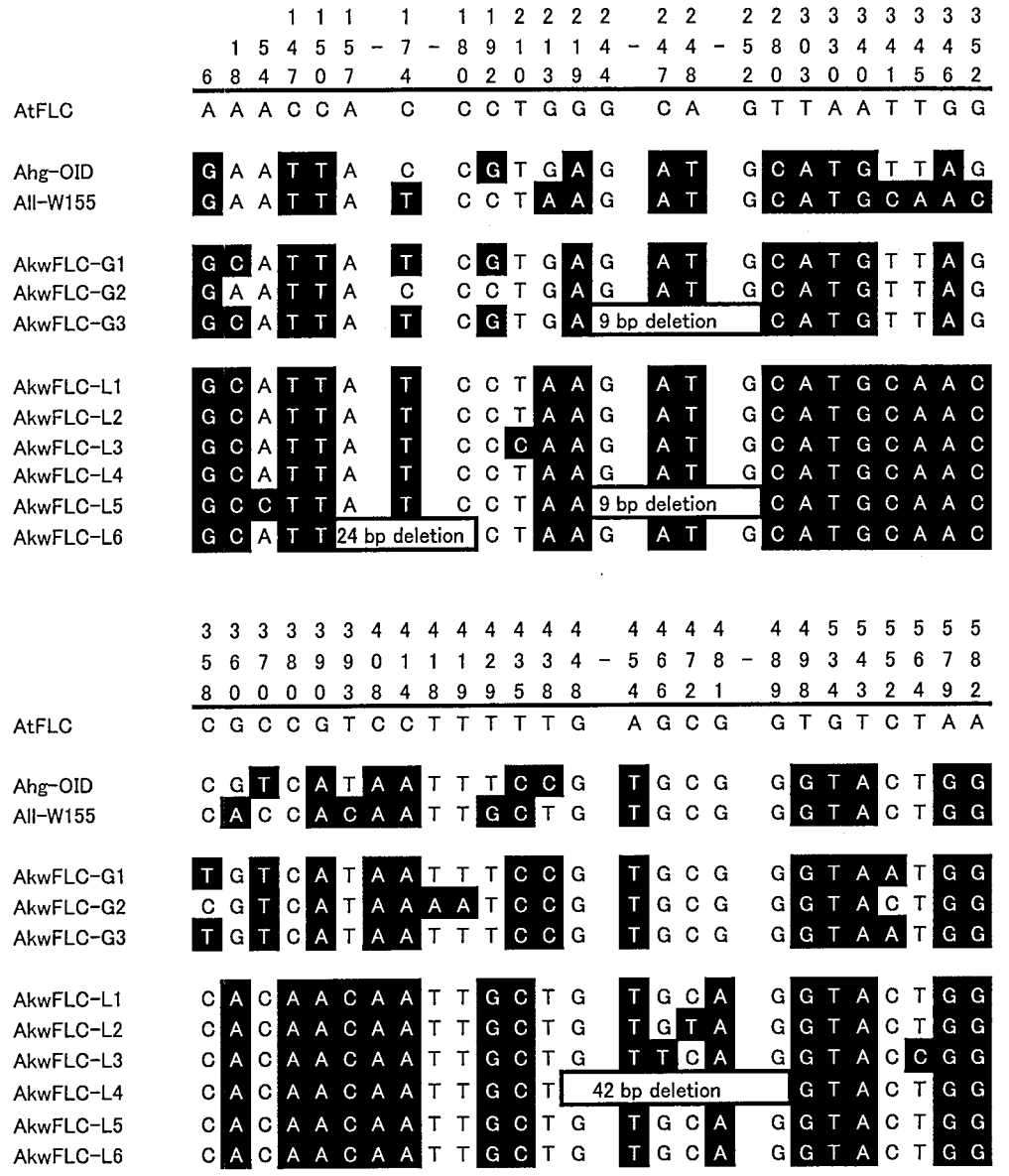

Fig. 4-3 Variation in nucleotide sequences of *AkwFLC*. Sequences of *Arabidopsis thaliana FLC, MAF1*, and two putative parental species, *A. lyrata* and *A. halleri* subsp. *gemmifera FLC* homologues are also listed.

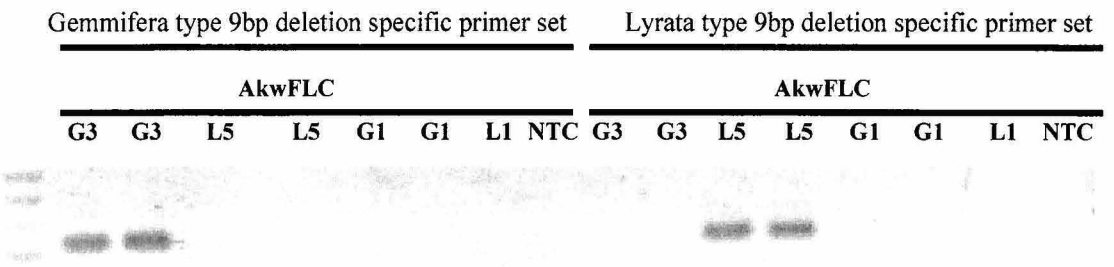

Fig. 4-4 Allele specific amplification of *AkwFLC* alleles using two sets of primer that were designed to detect *AkwFLC-G3* (gemmifera type with the 9 bp deletion) and *AkwFLC-L5* (lyrata type with the 9 bp deletion) alleles. G3, G1, L5, and L1 are the templates of gemmifera type with the 9 bp deletion, gemmifera type without the 9 bp deletion, lyrata type with the 9 bp deletion, and lyrata type without the 9 bp deletion, respectively.

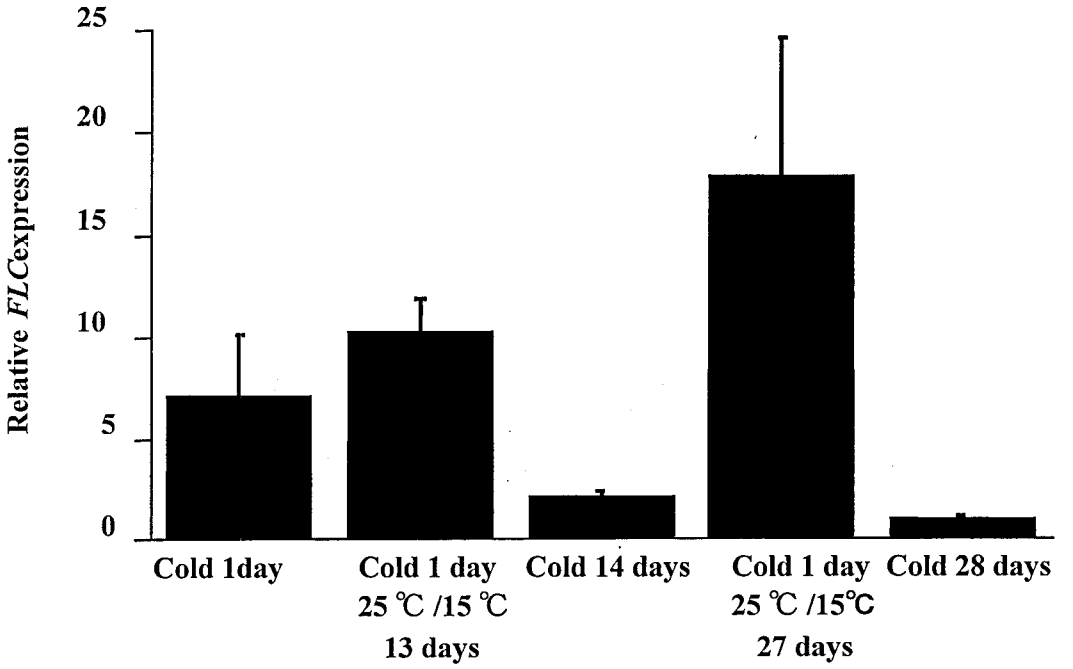

Fig. 4-5 Response to relative *AkwFLC* expression with different cold treatments. Bars indicate SD among three replicates.

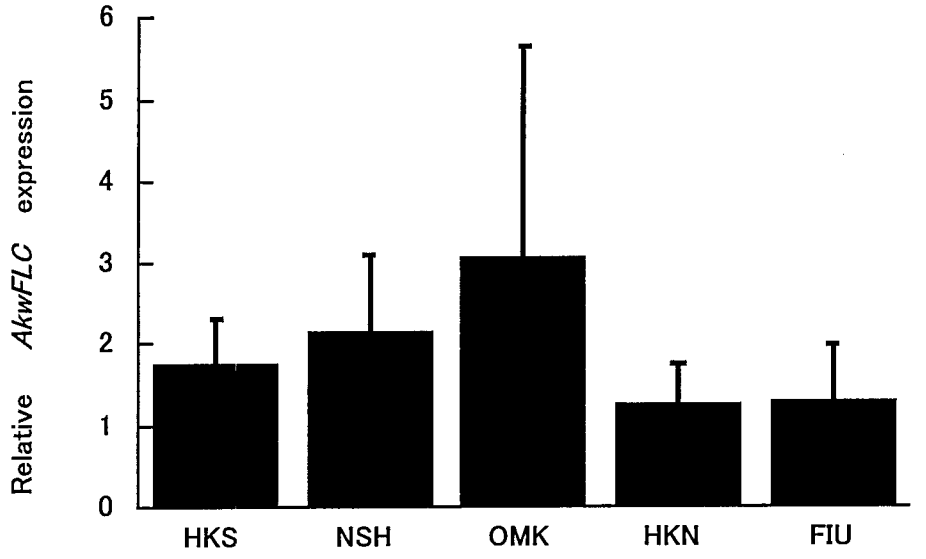

Fig. 4-6 The initial transcription levels of *AkwFLC* measured for seedlings from five natural populations. Average values for six strains per populations were shown. Bars indicate SD across strains.

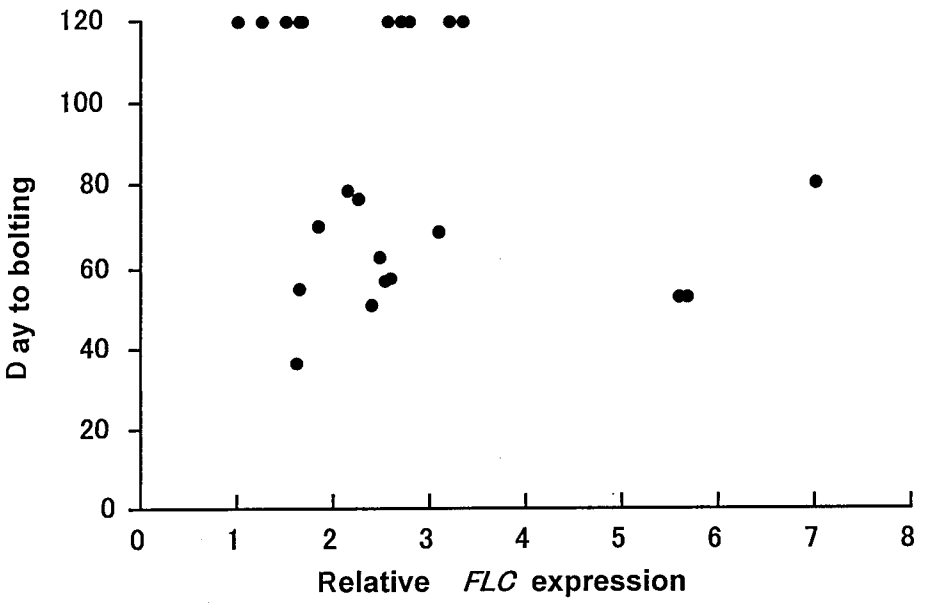

Fig. 4-7 Relationship between days to bolting under the non-vernalized condition and the initial *AkwFLC* transcription levels.

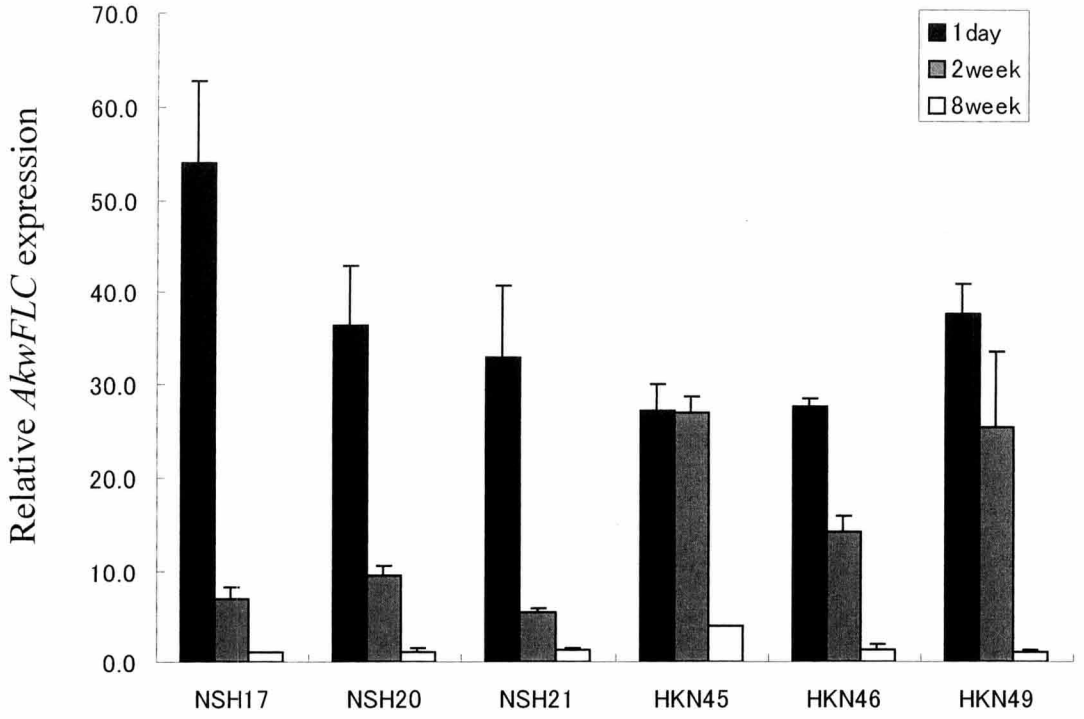

Fig.4-8 Responses of *AkwFLC* transcription to the different length of cold treatments for strains form the NSH and HKN populations. Three strains per populations were examined. Bars indicate SD of three replicates per strain. The two populations have contrasting flowering responses, and plants in NSH populations posses early flowering habit.