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Role of vernalization and of duplicated *FLOWERING LOCUS C* in the perennial *Arabidopsis lyrata*

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Summary

* *FLOWERING LOCUS C* (*FLC*) is one of the main genes influencing the vernalization requirement and natural flowering time variation in the annual *Arabidopsis thaliana*. Here we studied the effects of vernalization on flowering and its genetic basis in the perennial *Arabidopsis lyrata*.

* Two tandemly duplicated *FLC* genes (*FLC1* & *FLC2*) were compared with respect to the expression and DNA sequence. The effect of vernalization on flowering and on the expression of *FLC1* was studied in three European populations. The genetic basis of the *FLC1* expression difference between two of the populations was further studied by QTL mapping and sequence analysis.

* *FLC1* was shown to have a likely role in the vernalization requirement for flowering in *A. lyrata*. Its expression was decreased by vernalization and the northern study populations had a tendency to show higher *FLC1* expression than the southern one. QTL mapping between two of the populations revealed one QTL affecting *FLC1* expression in the genomic region containing the *FLC* genes. Most *FLC1* sequence differences between the study populations were found in the promoter region and in the first intron.

* Variation in the *FLC1* sequence may cause differences in *FLC1* expression between the late and early flowering *A. lyrata* populations.

Keywords: *FLC*, vernalization, *Arabidopsis lyrata*, flowering, gene expression, QTL, gene duplication

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Introduction

Populations of a plant species may have extensive ranges with highly variable environments with respect to temperature and the amount and quality of light (Munguia-Rosas *et al.*, 2011). Organisms living at high latitudes also experience seasons with highly contrasting environmental conditions. Synchronizing developmental transitions with the correct season is important for plants to reproduce successfully. In the model plant *Arabidopsis thaliana* many genetic pathways are known to mediate environmental signals and affect flowering (Ausin *et al.*, 2005). The importance of a particular pathway may differ between different environments and this plasticity can confer an evolutionary advantage (King & Heide, 2009). Among the flowering pathways, the vernalization pathway is important in many temperate species. The vernalization requirement ensures that plants do not flower in the fall when the environmental conditions are unfavorable for reproduction. The strength of the vernalization requirement can vary within plant species. For example, Norwegian high-latitude strawberry populations have an obligatory vernalization requirement, unlike other strawberry populations (Heide & Sønsteby, 2007). However, vernalization can accelerate flowering also in species and populations lacking the obligatory requirement (e.g. Méndez-Vigo *et al.*, 2011).

Plants need to be of a given age or size to be able to receive vernalization or other flowering promoting signals (Bernier & Perilleux, 2005). The length of the non-responsive period varies between species: for example, some grass species can receive a vernalization signal even at seed stage whereas other grasses become sensitive to vernalization when they are older (Heide, 1994 and references therein). In winter annual accessions of *A. thaliana* both seeds and rosettes respond to vernalization, but seed vernalization is more effective (Nordborg & Bergelson, 1999). Many perennials in the Brassicaceae, such as *Arabis alpina* and *Brassica oleracea*, become sensitive to vernalization after several weeks of growth (Lin *et al.*, 2005; Wang *et al.*, 2011).

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The genes *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) are important components of the vernalization pathway in *A. thaliana* (Johanson *et al.*, 2000; Sheldon *et al.*, 2000). Several studies have shown that these two genes may account for most of the observed flowering time variation among accessions of *A. thaliana* (Johanson *et al.*, 2000; Gazzani *et al.*, 2003; Shindo *et al.*, 2005), although some studies have indicated a more limited role in governing that variation (Scarcelli *et al.*, 2007; Brachi *et al.*, 2010). *FLC* is mostly expressed in leaves, in root tips and in the shoot apex (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). *FLC* encodes a MADS-box transcription factor that inhibits the expression of floral pathway integrator genes - a set of genes that combine information from flowering pathways and regulate the expression of flower meristem identity genes (Samach *et al.*, 2000; Simpson & Dean, 2002). Winter annual *A. thaliana* accessions germinate in the fall (Nordborg & Bergelson, 1999) and flowering is repressed before winter by high *FLC* expression. During the cold, *FLC* expression is reduced by epigenetic chromatin remodeling (Bastow *et al.*, 2004; Sung & Amasino, 2004) and in the spring plants have the competence to flower (Wilczek *et al.*, 2009)**,** but other environmental factors, such as day length and ambient temperature, still have effects on the timing of flowering. *FLC* expression is promoted by a transcription activation complex, in which *FRI* is one of the main components (Choi *et al.*, 2011), and inhibited by the genes of the autonomous and vernalization pathways (reviewed by Henderson & Dean, 2004; Wang *et al.*, 2012). Small regulatory RNAs transcribed from the *FLC* locus, COLDAIR from intron 1 and the antisense transcript COOLAIR covering the entire *FLC* locus, are also involved in *FLC* expression regulation (Swiezewski *et al.*, 2009; Heo & Sung, 2011). Genomic regions in the promoter and in the first intron are required for *FLC* down-regulation and maintenance of the low expression (Sheldon *et al.*, 2002; Sung *et al.*, 2006).

FLC and other flowering time genes have been examined extensively in the annual *A. thaliana* (e.g. Simpson & Dean, 2002; Srikanth & Schmid, 2011). *FLC* orthologues are known to mediate

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vernalization effects also in plant species with different life history strategies, such as in the biennial *B. oleracea* (Lin *et al.*, 2005) and in the polycarpic perennials *A. alpina* (Wang *et al.*, 2009) and *Arabidopsis halleri* (Aikawa *et al.*, 2010). In addition to mediating vernalization effects, the *FLC* orthologue in *A. alpina*, *PERPETUAL FLOWERING 1* (*PEP1*), was shown to have roles in limiting the duration of flowering and in governing the polycarpic flowering habit by regulating the fate of meristems (Wang *et al.*, 2009). Studies on the effect of *FLC* on flowering time variation among populations within the same species are needed to reveal the role of *FLC* in local adaptation.

FLC is triplicated in a short-lived perennial *Arabidopsis arenosa* and duplicated in *Arabidopsis lyrata* (Nah & Chen, 2010) due to sequential tandem duplications**.** In *A. arenosa*, the copies are differentially expressed, likely due to changes in the regulatory regions in the promoter (Wang *et al.*, 2006; Nah & Chen, 2010). The expression and the role of the two *FLC* genes in *A. lyrata* have not been studied, even though it was suggested that the two *FLC* genes in *A. lyrata* and *A. arenosa* have the same function as *FLC* in *A. thaliana* (Nah & Chen, 2010).

Here we report on the effects of vernalization on flowering and on the expression of the downstream copy of the two tandemly arranged *FLC* genes (*FLC1*) in Norwegian, Swedish and German *A. lyrata* populations. We have also studied the genetic basis of between-population differences in *FLC1* expression. We first examined the effects of seedling and rosette vernalization to find out whether both are effective in this species. Second, as the *FLC* gene is duplicated in *A. lyrata*, we made preliminary expression and sequence comparisons between the two *FLC* genes (*FLC1* and *FLC2*) in Norwegian and German populations. We showed that the downstream gene, *FLC1*, likely has the main role in the vernalization requirement in both populations, and then focused on this *FLC* gene. Third, we measured *FLC1* expression levels in Norwegian, Swedish and German *A. lyrata* populations before and after vernalization. We hypothesized that if *FLC1*

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mediates vernalization effects on flowering in *A. lyrata*, its expression will be decreased by vernalization. Further, since the Norwegian and Swedish populations flower later and respond more to vernalization than the German population (Kuittinen *et al.*, 2008), we expected to find higher *FLC1* expression in the northern populations. Fourth, in a QTL mapping experiment, we searched for genetic loci affecting *FLC1* expression differences between the German and the Norwegian populations. We specifically asked whether there is a QTL in the *FLC* or in the *FRI* genomic region. Finally, we examined the *FLC1* DNA sequence in the parental populations to detect causal variants for the observed QTL. On the basis of our QTL mapping results, we search for sequence differences at important regulatory regions.

Materials and Methods

Study populations

Arabidopsis lyrata (L.) O'Kane & Al-Shehbaz (Brassicaceae) is a perennial, polycarpic and outcrossing plant species, which has become a model plant for ecological genetics and genome evolution (Clauss & Mitchell-Olds, 2006; Hu *et al.*, 2011; Savolainen & Kuittinen, 2011). The species is divided in two subspecies, of which ssp. *petraea* inhabits Europe with fragmented distribution. Here, three *A. lyrata* ssp. *petraea* populations were studied: the Norwegian Spiterstulen (hereafter 'Sp', 61°38´N, 8°24´E), the German Plech ('Pl', 49°39´N, 11°29´E) and the Swedish Mjällom ('Mj' 65°55´N, 18°15´E; population Storsanden in Leinonen *et al.* (2009)). Many environmental factors differ between these locations, e.g. in Plech the thermal growing season (period from five successive days with daily-average temperature greater than +5.0°C to five successive days with daily-average temperature less than +5.0°C) is more than 150 days, whereas in Spiterstulen it is less than 50 days (Norwegian Meteorological Institute; Germany's National Meteorological Service; Quilot-Turion *et al.*, unpublished). These populations were chosen for this study because they represent the northern and southern parts of the distribution area of *A. lyrata* in

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Europe, because they have been shown to be locally adapted to their native environments (Leinonen *et al.*, 2009) and because they flower at different times in common garden experiments and respond differently to rosette vernalization (Riihimäki & Savolainen, 2004; Kuittinen *et al.*, 2008).

Effects of vernalization on flowering

We studied the effect of vernalization applied on plants at two different developmental stages. In the first experiment, newly germinated seeds from the three study populations were exposed to vernalization (seedling vernalization). In the second experiment, plants from the same populations experienced vernalization at four weeks age (rosette vernalization). Both experiments had controls that did not receive vernalization.

In the seedling vernalization experiment, 40 seeds from 10-20 families per population (generated by crosses in the lab) were germinated for 12 days on Petri dishes (+18^{o}C, LD 8:16), planted in pots with fertilized peat and gravel and vernalized for nine weeks (+4^{o}C, LD 8:16). Due to a timer failure the light was continuous in the cold room for a period of unknown length within the first 4.5 weeks of vernalization (and within the first 5.5 weeks of rosette vernalization). Since vernalization is effective both in short and in long days in *A. lyrata* (Kuittinen *et al.*, 2008; Leinonen *et al.*, unpublished) and since the photoperiod was correct for several weeks in the end of the 9 weeks vernalization, this should not have much influence on the results. Moreover, the plants in both vernalization experiments experienced the same photoperiod. After vernalization the pots were placed in the growth room (+22^{o}C, LD 20:4, Power Star HQI bulbs) together with germinated and planted seeds (40 per population) that had not received vernalization and served as controls. The vernalized and control plants were randomized within 8 blocks.

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In the rosette vernalization experiment, the similar seed material and the same growth and cold rooms were used as in the seedling vernalization experiment, and the experimental setup was identical, except that the plants were grown in a growth chamber (+22°C, LD 20:4) for 29 days before vernalization. Control plants were planted nine weeks later and were not exposed to vernalization. The set up of this experiment and the results for days to emergence of flower bud are briefly described in Kuittinen *et al.* (2008). Here we analyze additional traits from the same experiment and compare the results with those from the seedling vernalization experiment. Additional randomized plants for the expression study were included in this experiment; no phenotype data were collected from them since the apical meristem was collected for RNA extraction.

In both vernalization experiments, days to flowering (first open flower), excluding time in vernalization, was scored, as well as the number of leaves and the rosette size at flowering start. To find out whether the populations respond differently to vernalization treatments, the interaction between population and vernalization was studied with likelihood ratio tests between linear models (lm function in R; R Development Core Team) within each vernalization experiment. If the interaction term was not significant, the main effect of population and vernalization was also tested. Log_{10} transformation was used for traits whose residuals were not normally distributed. The block did not have a significant effect on any traits (Kruskal-Wallis tests, results not shown), and thus the data were analysed as a single randomized block. The likelihood ratio tests were also used to study the effect of rosette and seedling vernalization on the studied traits within each population. Chi square tests were used to study the effect of vernalization on flowering probability within the populations. The analyses were done in the statistical environment R v. 2.11.0 (R Development Core Team, 2010).

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Expression studies

***FLC1* expression in populations.** To examine whether *FLC1* expression is reduced by vernalization in *A. lyrata* and whether its expression differs between Pl, Sp and Mj populations, we studied *FLC1* expression by quantitative PCR (qPCR) in nonvernalized and vernalized plants in the rosette vernalization experiment. The primers (Table 1) were designed based on the *A. lyrata* genomic sequence and *FLC1* expression was normalized with that of the housekeeping gene *ß-TUBULIN6* (*TUB6*). When this and the QTL mapping experiment were conducted, it was not known that there are two *FLC* genes in *A. lyrata*. Afterwards, we found that the primers used here bound fully to *FLC1*, but there were 1 and 2 mismatches with *FLC2* in the forward and reverse primers. Moreover, when *FLC1* expression was measured both with the primers designed for this study and with the other set of primers designed specifically for *FLC1* in a set of samples from an unpublished experiment, the results were similar.

The apical meristem and the youngest leaves of 8 Pl, 10 Sp and 10 Mj plants before vernalization and of 12 Pl, 11 Sp and 9 Mj plants four days after vernalization were collected. After collection, the leaves were stored at -80°C and before RNA extraction 1 ml of RNA*later*®-ICE (Ambion) was added to each sample tube to protect RNA from degradation.

The apical meristem samples were disrupted by TissueLyser (Qiagen) and total RNA was isolated with RNeasy® Mini Kit (Qiagen) from the lysate, including DNase treatment (RNase-Free DNase Set, Qiagen), according to the manufacturer's instructions. To synthesize cDNA, 1 µg of RNA was incubated with 1 µl of random primers (Invitrogen, diluted to 0.03 µg/µl) and water (up to 9 µl) at +70°C for 10 minutes. 3 µl of 5x First Strand buffer (Invitrogen), 1.5 µl of 0.1 M DTT (Invitrogen), 1 µl of 10 mM dNTP mix and 0.5 µl of SuperScript III RT enzyme (Invitrogen) were added and reverse transcription reaction was incubated over night at +42°C. Dilution 1/100 was used for qPCR

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reactions, which were run with 7000 Sequence Detection System ver 3.0 (Applied Biosystems). On average three technical replicates were run. The template cDNA was amplified in 25 µl PCR reactions containing 5 µl of 1/100 cDNA dilution, 12.5 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 0.5 µl of ROX Reference Dye (Invitrogen), 0.5 µl of reverse and forward primer solutions (10 µM) and 6 µl of water with the following PCR program: 50°C for 2 minutes, 95°C for 2 minutes followed by 40 times 95°C for 15 seconds and 60°C for 1 minute. The correct product size and the lack of primer dimers were verified by gel electrophoresis.

The effect of rosette vernalization and population on *FLC1* expression were analyzed in R (v. 2.8.1; R Development Core Team, 2008) with analysis of covariance, where the response variable was *FLC1* Ct value (Cycle threshold) and explanatory variables *TUB6* Ct value, vernalization treatment (before/after vernalization) and population. The regression coefficient (0.51) of *TUB6* Ct value was taken into account when the *FLC1* expression was normalized for Figure 5 (normalized *FLC1* expression = 0.51 x Ct_{TUB6} - Ct_{FLC1}).

QTL mapping of *FLC1* expression. To identify the genomic regions explaining the difference in *FLC1* expression between the Sp and Pl populations, we did QTL mapping in a family of F2 plants. A random set of 123 F2 plants from a common garden experiment was used (see experiment 'GC05' in Quilot-Turion *et al.* (unpublished) for details of the crossing design and of the growing conditions). The growing conditions were similar to those described above for studying the effects of rosette vernalization; the only deviation was that day length during pre-growing was 14 hours. One young leaf from each plant was sampled at the age of four weeks, before the vernalization treatment. After collection, the samples were stored and treated as described above for population samples in the vernalization experiment until RNA extraction. Here RNA was isolated with RNeasy® 96 Kit (Qiagen) according to the isolation protocol for plant material, including DNase

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treatment. cDNA synthesis using 0.2 µg of RNA and qPCR were performed in a similar way as for the population samples. The *FLC1* expression for QTL mapping was normalized with that of *TUB6*, taking into account the regression coefficient 0.9 (normalized *FLC1* expression = 0.9 x Ct_{TUB6} - Ct_{FLC1}).

We used the linkage map that was constructed in Quilot-Turion *et al.* (unpublished) with 40 markers. The markers were microsatellites, or CAPS and dCAPS including some in flowering time genes, such as *FRI*. *FLC1* expression was normally distributed and the QTL analysis was done by genome-wide interval mapping with MapQTL® 5 (van Ooijen, 2004). A 5% LOD significance level for the whole genome was defined with 10 000 permutations.

***FLC1* and *FLC2* expressions comparison.** A subset of 72 F2 plants of the QTL mapping experiment was used for measuring *FLC1* and *FLC2* expressions to find out whether the Sp and Pl alleles of both loci are expressed and whether the two loci show similar expression patterns. The cDNA samples synthesized for QTL mapping described above were used and qPCR reactions were performed the same way. The correct product size and the lack of primer dimers were checked with a melting curve analysis. The expression levels were measured in 16-19 individuals from each genotype classes. To find out whether *FLC1* is differentially expressed in Pl and Sp homozygotes among the F2 plants, we tested the expression differences between these genotype classes as for population samples. The response variable was *FLC1* Ct value and the explanatory variables were *TUB6* Ct value and the genotype class (Pl_1Pl_2 or Sp_1Sp_2). Prior to analysis, the data were log_e transformed to make the distribution of the residuals closer to normal. When the expression levels of both genes were normalized for Figure 4, the regression coefficient 0.83 was used (normalized *FLC1*/*FLC2* expression = 0.83 x Ct_{TUB6} - $Ct_{FLC1/FLC2}$).

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Sequence analysis

To study the *FLC1* DNA sequence in the parental populations and to compare the sequence with that of *FLC2*, we sequenced the promoter and gene sequences of both *FLC* genes of two randomly chosen Sp and Pl individuals [*FLC1*: from -2894 to +5998 (sequence set 1); *FLC2*: from -832 to +6071 (sequence set 2); site information based on the published *A. lyrata* sequence (Hu *et al.*, 2011) and indicated from the start of the transcription start; for some individuals the obtained sequence was slightly shorter]. Due to technical issues, we did not obtain the full gene sequence of *FLC2* for both Pl individuals, and the Pl sequence was obtained by combining the sequences of the two individuals (individual Pl56: from -809 to +3707 and from +3809 to +6071; individual Pl59: from +3708 to +3808). To further study the *FLC1* sequence in the parental populations of the QTL mapping experiment, we also sequenced the full gene region of one of each F2-homozygote for parental alleles (genotype classes Pl_1Pl_2 and Sp_1Sp_2; sequence set 3). Note that these parental alleles represent two random alleles from each natural population. Finally, we studied the *FLC1* sequence polymorphism by sequencing a shorter region of 3529 bp, including 1134 bp from the end of the promoter, the first exon and 2210 bp from the beginning of intron 1 (from -1134 to +2395 bp) in 13 randomly chosen Pl and 7 Sp individuals (sequence set 4). Preliminary data indicated that the main differences between populations are found in this part.

To amplify the target sequences, we used Phusion DNA polymerase products and Piko Thermal Cycler (Finnzymes). The full promoter and gene region of *FLC1* was amplified in three and of *FLC2* in four overlapping fragments and the shorter *FLC1* sequence in one fragment using primer pairs designed based on the *A. lyrata* sequence (Table 2). The sequencing reactions were carried out with an ABI PRISM 3730 (Applied Biosystems) using the Big Dye Terminator kit v.3.1 (Applied Biosystems).

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Sequence data were verified and aligned with the CodonCodeAligner v. 3.5.7 (CodonCode Corporation, LI-COR, Inc) and edited with MEGA 4 (Tamura *et al.*, 2007). The sequences are deposited in the EMBL database under accession numbers XXXXXXXX-XXXXXXXX (sequence set 1), XXXXXXXX-XXXXXXXX (sequence set 2), XXXXXXXX-XXXXXXXX (sequence set 3) and XXXXXXXX-XXXXXXXX (sequence set 4).

We calculated the divergence between *A. lyrata FLC1* and *FLC2* at nonsynonymous (K_A) and synonymous (K_S) sites and their ratio (K_A/K_S; Nei & Gojobori, 1986; sequence sets 1 and 2). In addition, divergence between *FLC1* and *FLC2* from the *A. thaliana FLC* locus was studied separately. Some Pl individuals were excluded prior to analysis due to missing data. Before the analyses, the Pl and Sp sequences were pooled for each gene separately to gain one *A. lyrata* sequence for both genes. The level of polymorphism in *FLC1* within Sp and Pl populations was quantified separately using the whole *FLC1* gene sequences (sequence sets 1 and 3) and using the shorter sequences (sequence set 4) as the number of segregating sites (S) and as the average number of pairwise differences at silent sites (π; Tajima, 1983). The divergence between Pl and Sp populations was quantified, using the same sequence sets, as the average number of nucleotide substitutions per site between populations (Dxy; Nei, 1987), as F_{ST} (a measure of population differentiation based on mean pairwise differences; Hudson *et al.*, 1992), and as the number of fixed differences between populations. Summary statistics for sequence data were calculated using DnaSP v. 5.0 (Librado & Rozas, 2009).

Results

Effects of vernalization on flowering

Exposing plants to vernalization at seedling stage reduced days to flowering in Pl and Sp populations and leaf number at flowering in the Mj population. Though, rosette vernalization had a

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much bigger influence (Fig. 1a,b). When plants were vernalized at the rosette stage, they started flowering at a smaller size than the control plants. This effect was not seen when vernalization treatment was given to the seedlings (Fig. 1c). The seedling and rosette vernalization had contrasting effects on the flowering probability. In the Mj population, rosette vernalization increased and seedling vernalization decreased the probability. In the Pl and Sp populations, vernalization at neither stage affected the flowering probability (Fig. 1d).

The populations responded differently to rosette vernalization in all traits (significant vernalization x population interaction; Table 3). Vernalization had larger effect on the flowering time in the late flowering Mj and Sp populations than in the early flowering Pl population, so that after vernalization the time to flowering was very similar among populations (Figs. 1a, 2a). In contrast, all populations showed similar, small responses to vernalization that was given to the seedlings (Table 3; Figs. 1a, 2b). In addition to reducing the time to flowering, vernalization also reduced the variance of flowering time, but only when given to the rosettes (Fig. 2a,b).

FLC1 and FLC2 comparison

Comparing the sequence of *FLC1* to that of *FLC2* (sequence sets 1 and 2) showed that the genes show high similarity from about 350 bp before the first exon to the end of the gene. Upstream of this, the genes were so different that they could not be aligned. The exons of the genes were very similar: there were nine synonymous and seven nonsynonymous differences between *FLC1* and *FLC2* (Fig. 3b). Most of the nonsynonymous differences were detected in exon 4, whereas the synonymous changes were distributed more equally between the exons (Fig. 3b). An insertion of 1651 bp, introducing stop codons, was detected in exon 4 of *FLC2* in Spiterstulen (Fig. 3a,b). Intron 1 of *FLC1* was 880-891 bp shorter and intron 6 was 576-590 bp longer than those of *FLC2* (Fig. 3a), depending on the population. The divergences for nonsynonymous (K_A) and synonymous sites (K_S)

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between *FLC1* and *FLC2* were 0.016 and 0.073, respectively. A comparison of *FLC1* and *FLC2* with *A. thaliana FLC* showed that the divergence in these genes were quite similar based both on nonsynonymous and synonymous sites (K_A for *FLC1* = 0.034 and for *FLC2* = 0.028; K_S for *FLC1* = 0.120 and for *FLC2* = 0.115) and on K_A/K_S ratio (for *FLC1* = 0.283 and for *FLC2* = 0.243). The insertion in Sp *FLC2* was excluded from the sequence prior to the analysis.

The *FLC1* and *FLC2* expression comparison showed that both *FLC* genes were expressed in the populations, but they showed different expression patterns among the F2 genotype classes (Fig. 4a,b). Expression of *FLC1* was highest in Sp homozygotes (mean value for normalized *FLC1* expression: -6.67) and lowest in Pl homozygotes (-9.24; analysis of covariance, df = 1, F = 156.30, $P < 0.001$; Fig. 4a). Interestingly, the likely nonfunctional Sp alleles of *FLC2* were also expressed, but the Sp homozygotes showed the lowest expression. In the other genotype classes, *FLC2* was expressed at a higher and fairly similar level (Fig. 4b).

Effects of rosette vernalization and population on FLC1 expression

The overall effect of rosette vernalization was to decrease *FLC1* expression (analysis of covariance, df = 1, F = 28.27, $P < 0.001$, Fig. 5). Before vernalization plants from the northern Sp population had a tendency to show highest *FLC1* expression (mean value for normalized *FLC1* expression: -12.15) and plants from the southern Pl population the lowest (-13.06). After taking into account the effect of vernalization, the between-population difference in *FLC1* expression was close to significant (analysis of covariance, df = 2, F = 2.94, $P = 0.061$). When *FLC1* expression was analyzed separately within non-vernalized or vernalized plants, significant differences between populations were not detected in these small samples (df = 2, F = 2.43, $P = 0.109$ and df = 2, F = 1.00, $P = 0.380$, respectively).

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QTL mapping of FLC1 expression

We found one significant QTL with LOD score 19.41 explaining *FLC1* expression in the Pl x Sp F2 cross (Fig. 6). This QTL was located in the *FLC* gene region in chromosome 6 and explained 55.4% of the expression variation within the F2. Interestingly, possible *FRI* variation did not explain variation in *FLC1* expression in this cross, since we did not observe any indication of a QTL in chromosome 8 where *FRI* is located (Fig. 6). Moreover, no other QTL were found in the other parts of the genome. At the QTL peak, the *FLC1* expression of different genotype classes showed a pattern similar to the parental populations: the expression was highest in the Sp homozygotes (mean value for normalized *FLC1* expression: -2.63), lowest in Pl homozygotes (-3.70) and the expression of heterozygote classes was intermediate (-3.32 and -3.03).

FLC1 sequence analysis

The *FLC1* sequence of Pl and Sp (sequence sets 1 and 3) were very similar. The most notable difference between the Pl and Sp individuals were a 350 bp deletion in the promoter in Sp, but there were also several smaller differences (5 indels and 27 SNPs), mostly located in intron 1 (Fig. 7a). The most differentiated region of intron 1 was in the area from +425 bp to +1185 bp (sites compared to transcription start site in the Pl and Sp sequences). Further sequencing of 13 Pl and 7 Sp individuals (sequence set 4) indicated that the observed indels and SNPs are fixed in the study populations.

Overall, the nucleotide polymorphism at *FLC1* was lower in Sp than in Pl: the silent π was on average 5.5 times lower in Sp for the whole sequenced region (Table 4; Fig. 7b) and 15 times lower for the shorter sequenced region comprising the end of promoter, exon 1 and part of intron 1 (Table 4). Divergence, measured as Dxy was also highest in the same region where polymorphism varied

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mostly (Fig. 7a). Overall, F_{ST} between the populations was very high (0.62 for the whole gene and 0.85 for the shorter region; Table 4).

Discussion

Vernalization can have multiple effects in A. lyrata

Vernalization is known to have many effects on flowering in different taxa. For instance, vernalization has been shown to decrease heading/flowering time in *Phleum pratense* (Fiil *et al.*, 2011), in *A. thaliana* (Shindo *et al.*, 2006) and in *A. lyrata* (Kuittinen *et al.*, 2008) as well as flowering probability in *Bromus tectorum* (Meyer *et al.*, 2004). Here we showed that like in many perennials, adult *A. lyrata* plants responded more to vernalization than juvenile ones. Similar results were found when flowering responses were studied in three *A. lyrata* populations after seed vernalization and after overwintering as rosettes (Riihimäki & Savolainen, 2004), as natural winter increased the flowering probability more than seed vernalization. In contrast, vernalization given at seed stage is more effective than at rosette stage in *A. thaliana* (Nordborg & Bergelson, 1999) and seed and rosette vernalization were shown to have similar effects on flowering in the annual grass *B. tectorum* (Meyer *et al.*, 2004). Annual plants can invest most of the resources into flowering and reproduction, but for perennial plants it is important to flower after gaining enough resources for flowering as well as for forthcoming growing seasons. Vernalization also synchronizes flowering of the individuals of the same population, as, in this study, variation in flowering time was greatly reduced after rosette vernalization.

The FLC1 gene may have the main role in vernalization requirement in A. lyrata

It was recently suggested that all three *FLC* genes are functional in *A. arenosa* and that *FLC1* and *FLC2* genes in *A. arenosa* and *A. lyrata* share the same function as *FLC* in *A. thaliana* (Nah & Chen, 2010). Here we showed that there is a long insertion giving rise to premature stop codons in

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FLC2 in the *A. lyrata* plants from Spiterstulen, which indicates that *FLC2* is not functional in these individuals. The insertion was not observed in Pl sequence. However, it should be noted that we had sequence data for this area of *FLC2* only from two Sp and one Pl individuals and thus the insertion may be polymorphic in both populations. Apart from the insertion in *FLC2*, no other signs suggesting that this gene is non-functional were observed (K_A/K_S between the genes: 0.219). This suggests that the insertion is quite recent and additional deleterious changes have not accumulated yet. Separate comparison of *FLC1* and *FLC2* with *A. thaliana FLC* showed that *FLC1* may be slightly more diverged at nonsynonymous sites than *FLC2*, while synonymous divergence was about the same.

Based on our results the two *FLC* genes in *A. lyrata* have different expression patterns. In the F2 plants, the Sp alleles showed higher *FLC1* expression than Pl alleles, but for *FLC2* the results were opposite. If flowering time and vernalization requirement was regulated by an *FLC* gene in *A. lyrata*, it would be expected to have higher expression in the northern Sp population due to its stronger vernalization requirement compared to the more southern Pl population. Based on this reasoning, our results indicate that *FLC1* has the main role in governing the vernalization requirement for flowering in *A. lyrata*.

In addition to the vernalization response, *FLC* also has other functions in *A. thaliana*: it has been shown to affect flowering via the ambient temperature pathway, to be involved in the control of the circadian clock (reviewed by D'Aloia *et al.*, 2008) and of seed germination (Chiang *et al.*, 2009) and in many other developmental pathways (Scarcelli *et al.*, 2007; Deng *et al.*, 2011). It is thus possible that both *FLC* genes are functional at least in some populations of *A. lyrata*, but they are involved in different developmental pathways. Both genes may still share the same function, but they may be expressed in different tissues or their expression may be reduced compared to the

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progenitor gene and their combined expression may be important. This may maintain both duplicates of a gene as has been demonstrated in yeasts and in mammals (Qian *et al.*, 2010). However, gene duplicates maintaining the same function are quite rare. Silencing of one copy, partitioning of the tasks between the copies, or developing a new function for the other copy are more likely (e.g. Lynch *et al.*, 2001). As expression patterns differed between the *FLC* genes here, it seems possible that the genes are on their way to differentiate or that one of the copies is becoming a pseudogene. If the *FLC* duplicates have achieved different roles, it is possible that the functionally different gene regions may be in exon 4, where the genes are most clearly differentiated. Earlier it was estimated that the *FLC* duplication occurred about 2.5 MYA (Nah & Chen, 2010). Based on the current mutation rate estimate 7.0×10^{-9} (Ossowski *et al.*, 2010), *A. lyrata* and *A. thaliana* diverged about 10 MYA (Hu *et al.*, 2011) and thus the estimated time for the *FLC* duplication is about 5 MYA, which is a relatively short time in the evolution of a gene.

Flowering time differences between A. lyrata populations may be affected by differences in FLC1 expression

Variation in *FLC* expression has been shown to affect natural variation in flowering time in *A. thaliana* (Gazzani *et al.*, 2003; Lempe *et al.*, 2005) and also in other species in Brassicaceae, such as *Brassica napus* (Tadege *et al.*, 2001). The *FLC1* alleles from the early flowering southern Pl population had lower *FLC1* expression in non-vernalized F2 plants than alleles derived from the late flowering Sp population. The same trend was seen when *FLC1* expression was compared between Pl and Sp populations. After vernalization, we observed no differences in *FLC1* expression, meaning that the vernalization decreased the expression to similar level in the populations. Our results indicate that *FLC1* may be involved in the between population differences seen in flowering time and in vernalization requirement in *A. lyrata*, and thus could also be involved in local adaptation. Interestingly, three cabbage varieties having different vernalization requirements were

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shown to express *FLC* at similar level before vernalization, but the expression was decreased differentially between the varieties during vernalization (Lin *et al.*, 2005). This is in contrast to *A. lyrata*, where vernalization removed the initial differences. Thus, differences between populations/varieties in expression of *FLC* orthologues in the relatives of *A. thaliana* may be caused by different mechanisms in different species. The flowering time differences between populations of a plant species may be affected also by genes of the other flowering pathways, as was indicated by a gene expression profile study in *Capsella bursa-pastoris* (Huang *et al.*, 2012). However, in our experiment no difference in flowering time was observed when the adult plants were vernalized, indicating that the vernalization pathway genes play an important role in population-differentiation in flowering time in these conditions in *A. lyrata*.

In addition to *FLC*, there are also other vernalization-responsive genes in *A. thaliana* (Michaels & Amasino, 2001) that may be involved in the differentiation of the vernalization responses in natural populations. These include *VERNALIZATION INSENSITIVE 3* (Sung & Amasino, 2004), *VERNALIZATION 1* (Levy *et al.*, 2002) and *VERNALIZATION 2* (Gendall *et al.*, 2001). Flowering time variation in *A. thaliana* is mainly explained by several large-effect loci (e.g. Salomé *et al.*, 2011), for instance *FLC*, *FRI* and *MADS AFFECTING FLOWERING* (*MAF*) genes. It was previously shown that *FRI* and autonomous pathway genes balance each other to gain the appropriate level of *FLC* expression (Choi *et al.*, 2011). Here we found no indication that any upstream genes would be involved in population differentiation in *FLC* expression, since the only QTL was in the *FLC* region itself. However, in the QTL mapping experiment the results depend on the genotypes of the parents of the F2 cross. For example, it was shown earlier that a polymorphism in *FRI* affects flowering time variation within *A. lyrata* populations (Kuittinen *et al.*, 2008). However, this polymorphism did not segregate in the F2 cross we studied. We have also measured

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FRI expression in a subset of plants used for the population comparison in *FLC1* expression, but the preliminary results did not show any differences between these three particular populations.

Signs of directional selection in the regulatory regions of FLC1 in the northern Spiterstulen population

We found one QTL explaining the *FLC* expression difference between the parental populations in the genomic area containing the *FLC* genes. The QTL area was quite wide, but this result together with the sequence analysis suggest that the differential *FLC1* expression may be caused by changes in the regulatory region of *FLC1*.

Nucleotide variation was strongly reduced in Sp population and differentiation between Sp and Pl populations was increased, consistent with recent directional selection (Smith & Haigh, 1974). The observed F_{ST} (0.62) is much higher than F_{ST} between the Pl population and a Swedish Stubbsand population (0.2) based on almost 80 different loci (Ross-Ibarra *et al*., 2008). The 5.5-fold difference of neutral sequence variation in Sp compared with Pl highly exceeded the twofold reduction detected at neutral microsatellite loci (Muller *et al.*, 2008). Moreover, in the promoter region, exon 1 and part of intron 1, the populations diverged more compared to other regions of the *FLC1* gene (variation reached 15-fold difference). Further, these regions were also highly divergent compared to the corresponding regions in *A. thaliana* (i.e. they were impossible to align), which can be interpreted as high mutation rate at these regions. Thus, low variation in the promoter region and in the first intron in Sp is in contrast to divergence. Hence, it is possible that these expression regulating regions of *FLC1* have been under directional selection in recent history.

Previous studies in *A. thaliana* have identified regions important for *FLC* expression in non-vernalized plants particularly in the promoter. Moreover, regions affecting both the *FLC* repression

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during cold and the maintenance of the repression at normal temperatures have been located both to the promoter and the first intron (Sheldon *et al.*, 2002). For instance, a protein complex including FRI binds to the *FLC* promoter and leads to an active chromatin state and high expression (Choi *et al.*, 2011). Furthermore, Choi *et al.* (2011) identified a 15 bp binding site sequence for one of the complex components (SUF1) at -363 to about -331 from the transcription start site of *FLC*. A similar motif (differing by two substitutions) was identified in the promoter sequence of *FLC1* from the Pl population. This putative motif is located in the region where the Sp *FLC1* sequence has a 350 bp deletion and thus lacks the motif. However, as the motif in *A. thaliana* confers high expression, the expectation would be that the Sp deletion would lead to lower *FLC1* expression, which is not supported by data from this study. Clearly, functional studies of the promoter and intron sequence variation observed in *FLC1* from Sp and Pl are needed to clarify their effect on *FLC1* regulation. Population genomic analysis of these regions can also give cues on the importance of the different loci.

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Figure legends

Fig. 1 Effects of rosette (left) and seedling vernalization (right) on (a) days to flowering, (b) leaf number at flowering start, (c) rosette size at flowering start, and (d) flowering probability, in German Plech (Pl), Norwegian Spiterstulen (Sp) and Swedish Mjällom (Mj) *Arabidopsis lyrata*

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populations. Mean values are shown with 95% confidence intervals. Control plants are shown by white bars and vernalized plants by grey bars. Significant differences between vernalization and control treatments within populations (flowering probability: X^2 test; other traits: likelihood ratio tests between linear models) are indicated with asterisks. *) $P < 0.05$, **) $P < 0.01$, ***) $P < 0.001$, ns) $P > 0.05$, na) not analyzed due to low number of observations.

Fig. 2 Distributions of timing of flowering in *Arabidopsis lyrata* populations from Plech (Pl), Spiterstulen (Sp) and Mjällom (Mj) in (a) rosette and (b) seedling vernalization experiments. Upper row controls, lower row vernalized. The medians are shown by the arrowheads. Note the different scales on the y-axis.

Fig. 3 (a) Structure of *FLOWERING LOCUS C 1* and *2* (*FLC1* and *FLC2*) genes in *Arabidopsis lyrata*. Open boxes indicate the promoter and the 3´untranslated regions, closed boxes exons and solid line introns. (b) Exons (E1 - E7) of *FLC1* and *FLC2* in *A. lyrata*. Nonsynonymous substitutions are shown by open circles and synonymous substitutions by closed circles. *FLC* gene, in which the substitution has occurred, was determined by comparing the DNA sequence with that of *Arabidopsis thaliana*. Location of the 1651 bp insertion in Spiterstulen (Sp) population is indicated in both figures, but note that the insertion is not to scale in figure b. Sequence sets 1 and 2 (see Materials and Methods for details) were used in these figures.

Fig. 4 (a) *FLOWERING LOCUS C 1* (*FLC1)* and (b) *FLOWERING LOCUS C 2* (*FLC2*) expression in Spiterstulen (Sp) and Plech (Pl) homozygotes and two heterozygotes for parental alleles of Sp x Pl F2 population of *Arabidopsis lyrata* for QTL mapping. Expression levels were measured in the samples collected before the vernalization by qPCR and normalized with expression of *TUBULIN6* (*TUB6*), using the regression coefficient 0.83 (normalized *FLC1*/*FLC2* expression = 0.83 x Ct_{TUB6} -

Ct$_{FLC1/FLC2}$). The median is indicated by thick line, lower and upper quartiles by boxes and outliers by dots. The significance of *FLC1* expression difference between Pl and Sp homozygotes was analyzed with analysis of covariance.

Fig. 5 Expression levels of *FLOWERING LOCUS C 1* (*FLC1*) in Plech, Spiterstulen and Mjällom populations of *Arabidopsis lyrata* before and after vernalization of nine weeks. The expression was measured by qPCR and normalized with expression of *TUBULIN6* (*TUB6*) and the regression coefficient 0.51 was taken into account (normalized *FLC1* expression = 0.51 x Ct$_{TUB6}$ - Ct$_{FLC1}$). The median is indicated by thick line, lower and upper quartiles by boxes and outlier by dot.

Fig. 6 One QTL for *FLOWERING LOCUS C 1* (*FLC1*) expression before the vernalization was found in the linkage group 6 in the gene region containing the *FLC* genes (*FLC1* and *2*; location shown) in F2 cross between German Plech and Norwegian Spiterstulen populations of *Arabidopsis lyrata*. The LOD score is shown by solid line and 5% LOD significance level (3.6) by dashed line. No QTL was found in linkage group 8 (or any other linkage groups), where *FRIGIDA* (*FRI*), an important regulator of *FLC*, is located.

Fig. 7 (a) Divergence, measured as Dxy, between Spitertulen and Plech populations of *Arabidopsis lyrata* in *FLOWERING LOCUS C 1* (*FLC1*). (b) Sliding window for silent nucleotide diversity (π) across *FLC1* in *Arabidopsis lyrata* populations from Plech (solid line) and Spiterstulen (dashed line). Analyses are based on sequence sets 1 and 3 (see Materials and Methods for details). The structure of the *FLC1* gene is shown below the both figures (generated using Exon-Intron Graphic maker by Nikhil Bhatla, www.wormweb.org/exonintron). Open boxes indicate the promoter and 3' untranslated regions, closed boxes exons and solid line introns. The 350 bp deletion in the promoter in Spiterstulen population is shown by a thick horizontal black bar.

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Fig. 1

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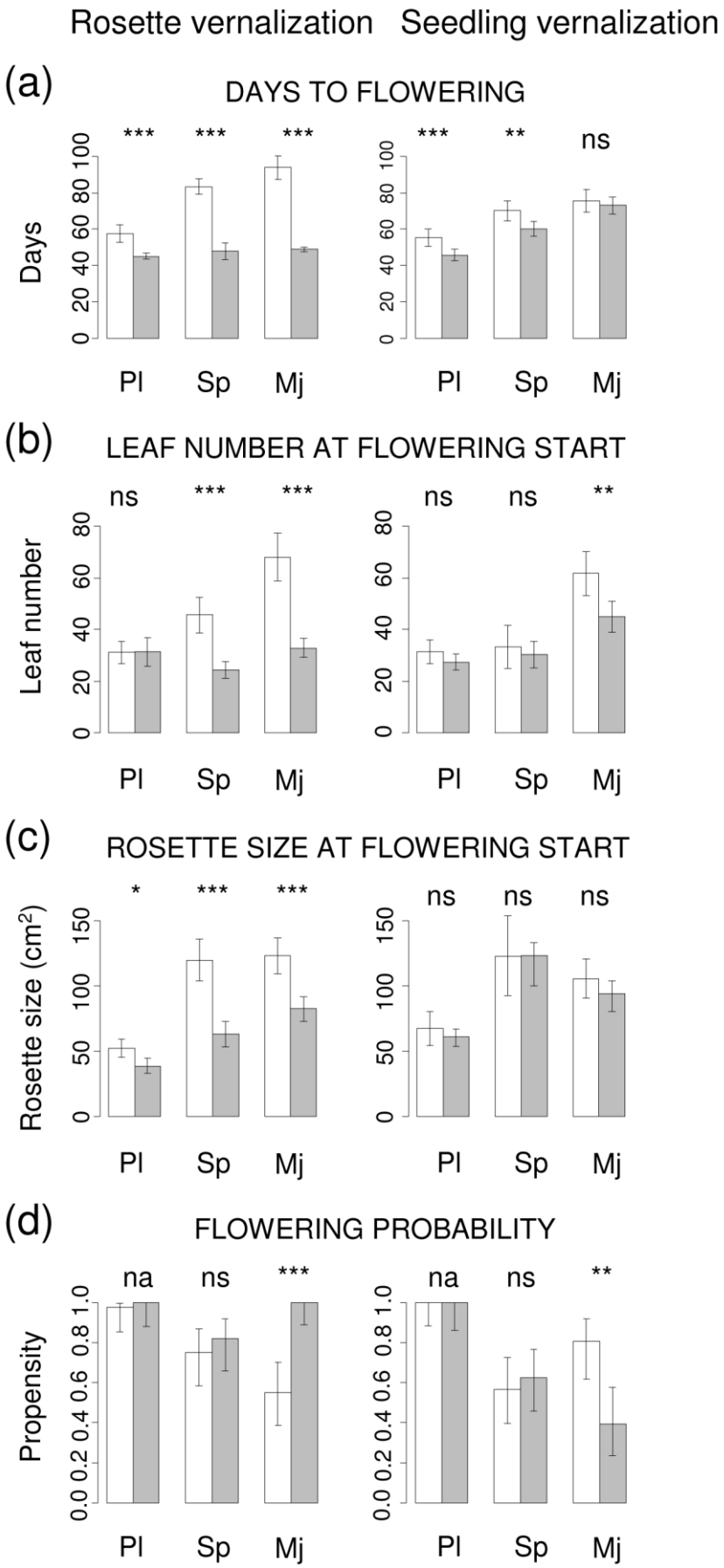

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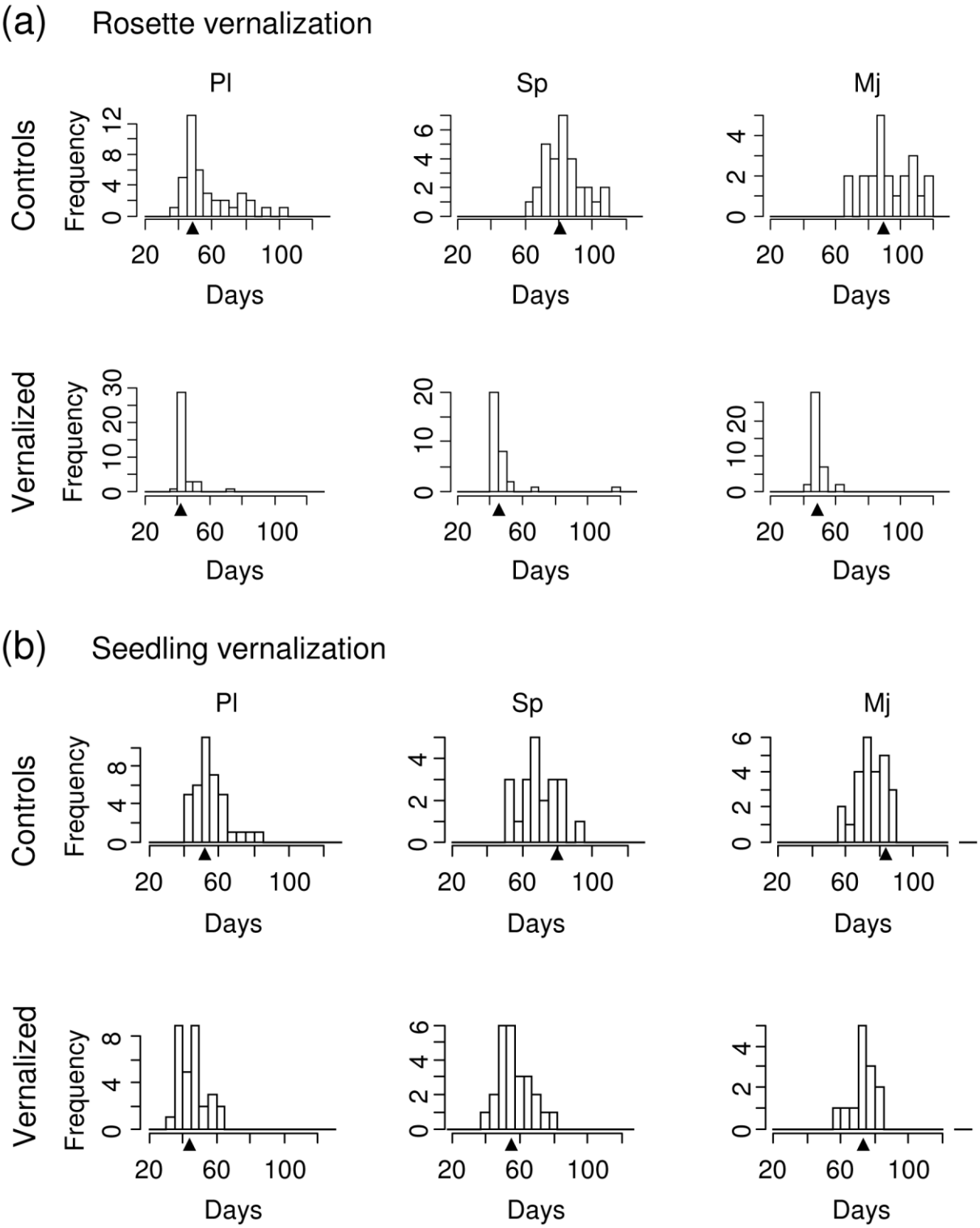

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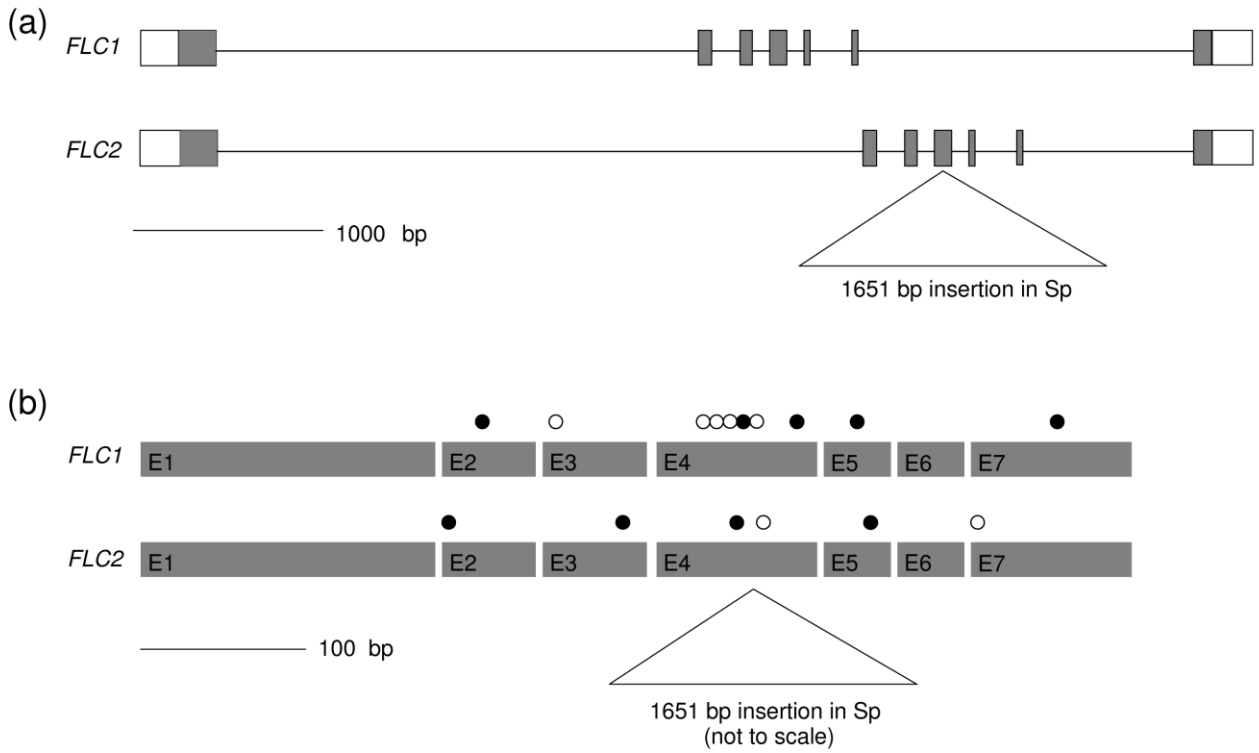

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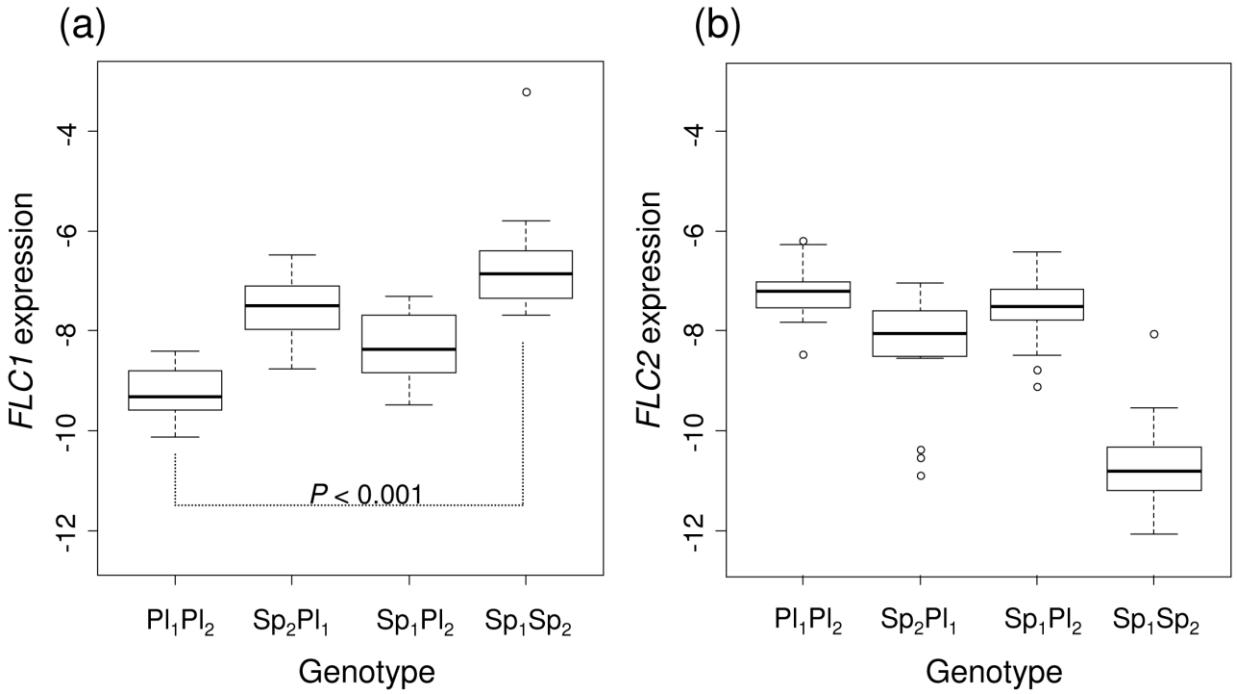

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843 Fig. 5

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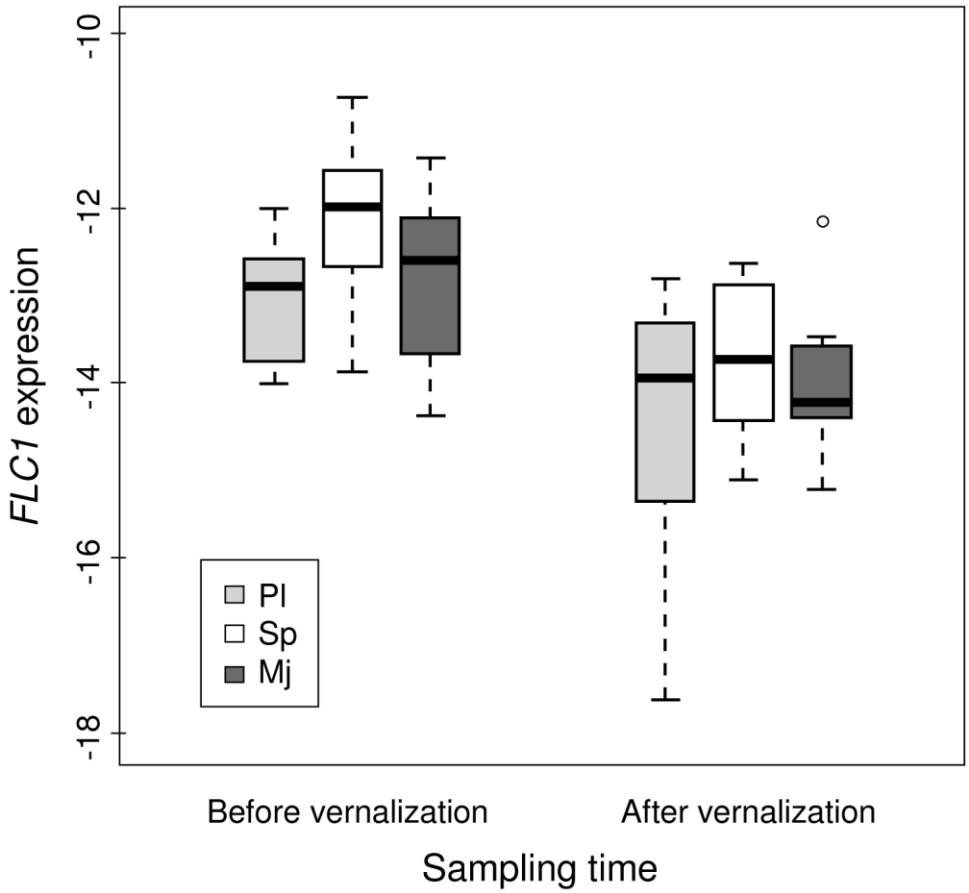

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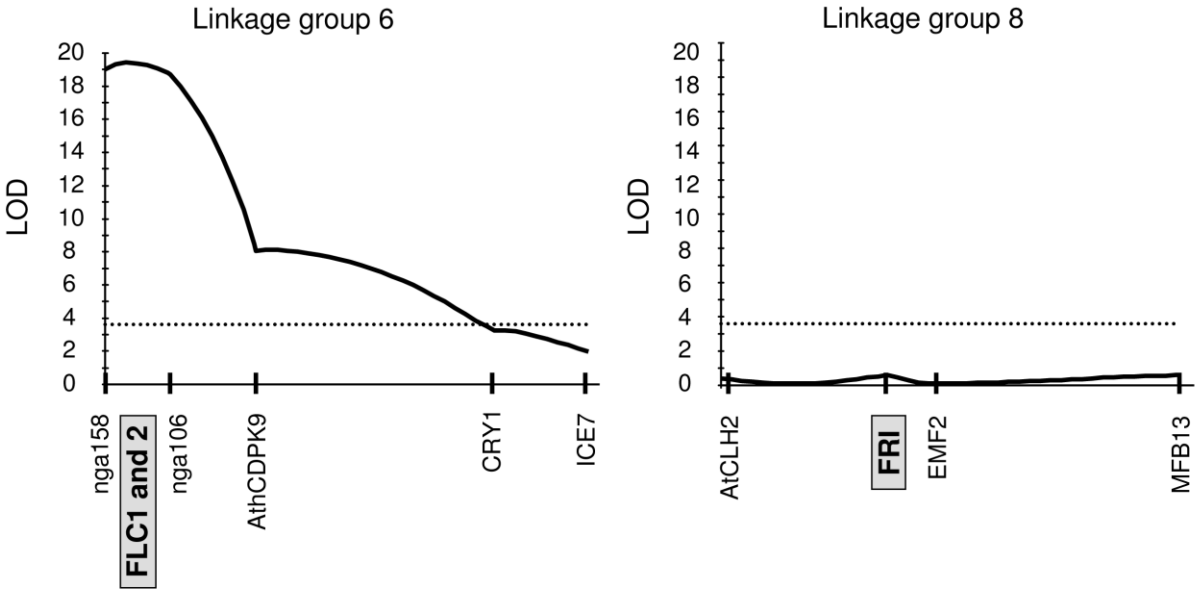
845 Fig. 6



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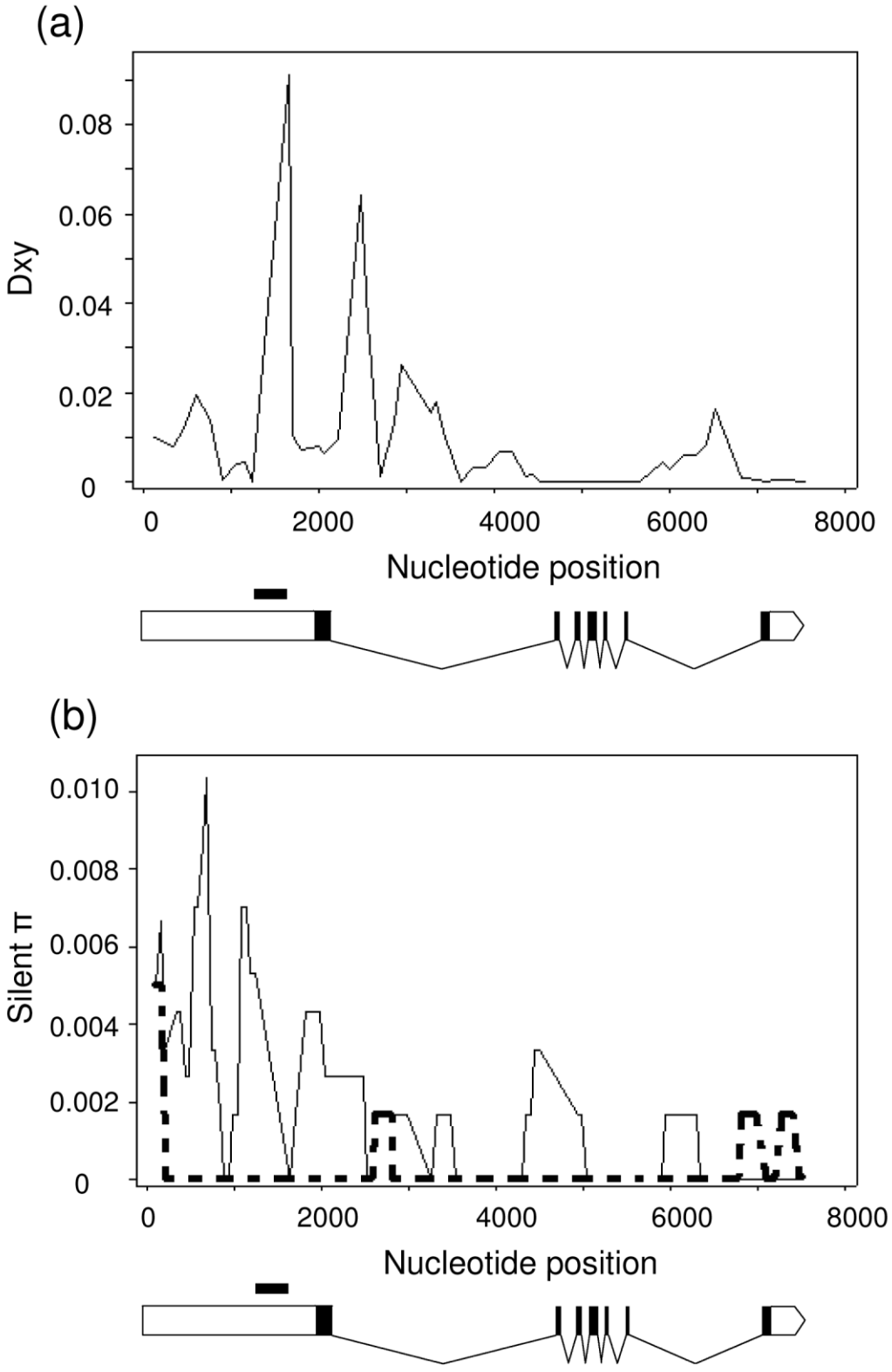

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Table 1 qPCR primers used for amplifying *FLOWERING LOCUS C 1* and *2* (*FLC1* and *FLC2*) and *ß-TUBULIN6* (*TUB6*) genes in *Arabidopsis lyrata* in three experiments: *FLC1*pop = *FLC1* expression in populations; QTL = QTL mapping of *FLC1* expression; *FLC*comp = *FLC1* and *FLC2* expression comparison.

Experiment	Gene	Forward primer	Reverse primer
FLC1 pop, QTL	*FLC1*	CATGAGCTACTTGAACTTGTGGAAA	TTCGGCACTCACATTATTGACAT
FLC comp	*FLC1*	GAGTGCCGAAACTCTTCTTCAACTA	GCCAAAACCTGGTTCTCTTCT
FLC comp	*FLC2*	AGCAAGCTTGTGGGATCAAA	CCTCCAATTGAACAAGAGTATCGA
FLC1 pop, QTL, *FLC* comp	*TUB6*	ACCACTCCTAGCTTTGGTGATCTG	AGGTTCACTGCGAGCTTCCTCA

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Table 2 Primer pairs used for amplifying *FLOWERING LOCUS C 1* and *2* (*FLC1* and *FLC2*) genomic DNA (*FLC1*: from -2894 to +5998; *FLC2*: from -832 to +6071) and a shorter sequence of *FLC1* (from -1134 to +2395) in two populations of *Arabidopsis lyrata*. Whole *FLC1* gene was amplified in three overlapping fragment, a shorter *FLC1* sequence in one fragment and *FLC2* gene in four fragments.

Gene	Forward primer	Reverse primer
FLC1	TGAGTCAGGAACGAGTCACG	CTTGTCGGCTACTTTTGT
	CATTTACCAAAGAAAGGTAATGC	TGTAAACGCAGCCTCAATCTT
	CCCATGTCATCACTTTGTGG	TCCAACCATTCGCGTTTATT
FLC1 shorter	GTTAGCTTTCCGCCAGTTTG	TGTAAACGCAGCCTCAATCTT
FLC2	AACGAGCAAATGAATGCAAA	AATATATTGGAGGGTTGTAGTAACA
	AATCTTCAGTTTTGTGCTCTTTACTT	CACACTGTCTAACCCCGAGT
	TGGGAAGTCTAGGCTTTGGTT	GCCAAAACCTGGTTCTCTTCT
	TTTGCAACTACTTCCCAATGC	CAAACGCTCGCCCTTATCA

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Table 3 Effect of vernalization, *Arabidopsis lyrata* population and their interaction on traits of interest in rosette and seedling vernalization experiments, from the likelihood ratio tests between linear models. a) Log_{10} transformation.

	Vernalization x population			Vernalization			Population		
Rosette vernalization	df	F	*P*	df	F	*P*	df	F	*P*
Days to flowering (a)	2	34.67	< 0.001						
Leaf number at flowering start (a)	2	19.06	< 0.001						
Rosette size at flowering start (a)	2	4.07	0.018						
Seedling vernalization	df	F	*P*	df	F	*P*	df	F	*P*
Days to flowering	2	2.11	0.124	1	28.90	< 0.001	2	87.02	< 0.001
Leaf number at flowering start (a)	2	1.37	0.257	1	8.55	0.004	2	58.55	< 0.001
Rosette size at flowering start (a)	2	0.16	0.856	1	0.02	0.887	2	23.57	< 0.001

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Table 4 Summary statistics for polymorphism and divergence in *FLOWERING LOCUS C 1* in Spiterstulen (Sp) and Plech (Pl) population of *Arabidopsis lyrata*. a) Set 1: randomly chosen Pl and Sp individuals; Set 3: F2 plants (from Pl x Sp cross) homozygous for parental alleles; Set 4: randomly chosen Pl and Sp plants; See Materials and Methods for details, b) Length (bp), c) Number of segregating sites, d) Pariwise nucleotide heterozygosity, e) See Materials and Methods for details.

				Polymorphism within populations		Divergence between populations (e)		
Population	Sequence set (a)	L (b)	N	Silent S (c)	Silent π (d)	Dxy	F_{st}	N of fixed differences
Pl	1 and 3	8892	3	27	0.00192	0.007	0.62	27
Sp	1 and 3	8892	3	7	0.00035			
Pl	4	3529	13	11	0.00164	0.013	0.85	19
Sp	4	3529	7	1	0.00011			