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► **To cite this version:**

Ulla Kemi, Anne Niittyvuopio, Tuomas Toivainen, Anu Pasanen, Bénédicte Quilot-Turion, et al.. Role of vernalization and of duplicated FLOWERING LOCUS C in the perennial *Arabidopsis lyrata*. *New Phytologist*, 2013, 197 (1), pp.323-335. 10.1111/j.1469-8137.2012.04378.x . hal-02645614

HAL Id: hal-02645614

<https://hal.inrae.fr/hal-02645614>

Submitted on 29 May 2020

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

3
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26 **Word counts:**

27 Summary: 200 (max 200)

28 Total: 6499

29 Introduction: 1138

30 Materials and Methods: 2366

31 Results: 1044

32 Discussion: 1830

33 Acknowledgements: 121

34

35 **Number of figures: 7**

36 **Number of tables: 4**

37

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51 Summary

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

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

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

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

69

70

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

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76 Introduction

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

91
92 Plants need to be of a given age or size to be able to receive vernalization or other flowering
93 promoting signals (Bernier & Perilleux, 2005). The length of the non-responsive period varies
94 between species: for example, some grass species can receive a vernalization signal even at seed
95 stage whereas other grasses become sensitive to vernalization when they are older (Heide, 1994 and
96 references therein). In winter annual accessions of *A. thaliana* both seeds and rosettes respond to
97 vernalization, but seed vernalization is more effective (Nordborg & Bergelson, 1999). Many
98 perennials in the Brassicaceae, such as *Arabis alpina* and *Brassica oleracea*, become sensitive to
99 vernalization after several weeks of growth (Lin *et al.*, 2005; Wang *et al.*, 2011).

100

101 The genes *FLOWERING LOCUS C (FLC)* and *FRIGIDA (FRI)* are important components of the
102 vernalization pathway in *A. thaliana* (Johanson *et al.*, 2000; Sheldon *et al.*, 2000). Several studies
103 have shown that these two genes may account for most of the observed flowering time variation
104 among accessions of *A. thaliana* (Johanson *et al.*, 2000; Gazzani *et al.*, 2003; Shindo *et al.*, 2005),
105 although some studies have indicated a more limited role in governing that variation (Scarcelli *et al.*,
106 2007; Brachi *et al.*, 2010). *FLC* is mostly expressed in leaves, in root tips and in the shoot apex
107 (Michaels & Amasino, 1999; Sheldon *et al.*, 1999). *FLC* encodes a MADS-box transcription factor
108 that inhibits the expression of floral pathway integrator genes - a set of genes that combine
109 information from flowering pathways and regulate the expression of flower meristem identity genes
110 (Samach *et al.*, 2000; Simpson & Dean, 2002). Winter annual *A. thaliana* accessions germinate in
111 the fall (Nordborg & Bergelson, 1999) and flowering is repressed before winter by high *FLC*
112 expression. During the cold, *FLC* expression is reduced by epigenetic chromatin remodeling
113 (Bastow *et al.*, 2004; Sung & Amasino, 2004) and in the spring plants have the competence to
114 flower (Wilczek *et al.*, 2009), but other environmental factors, such as day length and ambient
115 temperature, still have effects on the timing of flowering. *FLC* expression is promoted by a
116 transcription activation complex, in which *FRI* is one of the main components (Choi *et al.*, 2011),
117 and inhibited by the genes of the autonomous and vernalization pathways (reviewed by Henderson
118 & Dean, 2004; Wang *et al.*, 2012). Small regulatory RNAs transcribed from the *FLC* locus,
119 COLDAIR from intron 1 and the antisense transcript COOLAIR covering the entire *FLC* locus, are
120 also involved in *FLC* expression regulation (Swiezewski *et al.*, 2009; Heo & Sung, 2011). Genomic
121 regions in the promoter and in the first intron are required for *FLC* down-regulation and
122 maintenance of the low expression (Sheldon *et al.*, 2002; Sung *et al.*, 2006).
123
124 *FLC* and other flowering time genes have been examined extensively in the annual *A. thaliana* (e.g.
125 Simpson & Dean, 2002; Srikanth & Schmid, 2011). *FLC* orthologues are known to mediate

126 vernalization effects also in plant species with different life history strategies, such as in the biennial
127 *B. oleracea* (Lin *et al.*, 2005) and in the polycarpic perennials *A. alpina* (Wang *et al.*, 2009) and
128 *Arabidopsis halleri* (Aikawa *et al.*, 2010). In addition to mediating vernalization effects, the *FLC*
129 orthologue in *A. alpina*, *PERPETUAL FLOWERING 1 (PEP1)*, was shown to have roles in limiting
130 the duration of flowering and in governing the polycarpic flowering habit by regulating the fate of
131 meristems (Wang *et al.*, 2009). Studies on the effect of *FLC* on flowering time variation among
132 populations within the same species are needed to reveal the role of *FLC* in local adaptation.
133
134 *FLC* is triplicated in a short-lived perennial *Arabidopsis arenosa* and duplicated in *Arabidopsis*
135 *lyrata* (Nah & Chen, 2010) due to sequential tandem duplications. In *A. arenosa*, the copies are
136 differentially expressed, likely due to changes in the regulatory regions in the promoter (Wang *et al.*,
137 2006; Nah & Chen, 2010). The expression and the role of the two *FLC* genes in *A. lyrata* have not
138 been studied, even though it was suggested that the two *FLC* genes in *A. lyrata* and *A. arenosa* have
139 the same function as *FLC* in *A. thaliana* (Nah & Chen, 2010).
140
141 Here we report on the effects of vernalization on flowering and on the expression of the
142 downstream copy of the two tandemly arranged *FLC* genes (*FLC1*) in Norwegian, Swedish and
143 German *A. lyrata* populations. We have also studied the genetic basis of between-population
144 differences in *FLC1* expression. We first examined the effects of seedling and rosette vernalization
145 to find out whether both are effective in this species. Second, as the *FLC* gene is duplicated in *A.*
146 *lyrata*, we made preliminary expression and sequence comparisons between the two *FLC* genes
147 (*FLC1* and *FLC2*) in Norwegian and German populations. We showed that the downstream gene,
148 *FLC1*, likely has the main role in the vernalization requirement in both populations, and then
149 focused on this *FLC* gene. Third, we measured *FLC1* expression levels in Norwegian, Swedish and
150 German *A. lyrata* populations before and after vernalization. We hypothesized that if *FLC1*

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

160

161 **Materials and Methods**

162 *Study populations*

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

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

179

180 *Effects of vernalization on flowering*

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

186

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

199

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

210

211 In both vernalization experiments, days to flowering (first open flower), excluding time in
212 vernalization, was scored, as well as the number of leaves and the rosette size at flowering start. To
213 find out whether the populations respond differently to vernalization treatments, the interaction
214 between population and vernalization was studied with likelihood ratio tests between linear models
215 (lm function in R; R Development Core Team) within each vernalization experiment. If the
216 interaction term was not significant, the main effect of population and vernalization was also tested.
217 Log₁₀ transformation was used for traits whose residuals were not normally distributed. The block
218 did not have a significant effect on any traits (Kruskal-Wallis tests, results not shown), and thus the
219 data were analysed as a single randomized block. The likelihood ratio tests were also used to study
220 the effect of rosette and seedling vernalization on the studied traits within each population. Chi
221 square tests were used to study the effect of vernalization on flowering probability within the
222 populations. The analyses were done in the statistical environment R v. 2.11.0 (R Development
223 Core Team, 2010).

224

225 *Expression studies*

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

237
238 The apical meristem and the youngest leaves of 8 Pl, 10 Sp and 10 Mj plants before vernalization
239 and of 12 Pl, 11 Sp and 9 Mj plants four days after vernalization were collected. After collection,
240 the leaves were stored at -80°C and before RNA extraction 1 ml of *RNAlater*®-ICE (Ambion) was
241 added to each sample tube to protect RNA from degradation.

242
243 The apical meristem samples were disrupted by TissueLyser (Qiagen) and total RNA was isolated
244 with RNeasy® Mini Kit (Qiagen) from the lysate, including DNase treatment (RNase-Free DNase
245 Set, Qiagen), according to the manufacturer's instructions. To synthesize cDNA, 1 µg of RNA was
246 incubated with 1 µl of random primers (Invitrogen, diluted to 0.03 µg/µl) and water (up to 9 µl) at
247 +70°C for 10 minutes. 3 µl of 5x First Strand buffer (Invitrogen), 1.5 µl of 0.1 M DTT (Invitrogen),
248 1 µl of 10 mM dNTP mix and 0.5 µl of SuperScript III RT enzyme (Invitrogen) were added and
249 reverse transcription reaction was incubated over night at +42°C. Dilution 1/100 was used for qPCR

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

257

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

264

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

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

279

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

285

286 ***FLC1* and *FLC2* expressions comparison.** A subset of 72 F2 plants of the QTL mapping
287 experiment was used for measuring *FLC1* and *FLC2* expressions to find out whether the Sp and Pl
288 alleles of both loci are expressed and whether the two loci show similar expression patterns. The
289 cDNA samples synthesized for QTL mapping described above were used and qPCR reactions were
290 performed the same way. The correct product size and the lack of primer dimers were checked with
291 a melting curve analysis. The expression levels were measured in 16-19 individuals from each
292 genotype classes. To find out whether *FLC1* is differentially expressed in Pl and Sp homozygotes
293 among the F2 plants, we tested the expression differences between these genotype classes as for
294 population samples. The response variable was *FLC1* Ct value and the explanatory variables were
295 *TUB6* Ct value and the genotype class (Pl₁Pl₂ or Sp₁Sp₂). Prior to analysis, the data were log_e
296 transformed to make the distribution of the residuals closer to normal. When the expression levels
297 of both genes were normalized for Figure 4, the regression coefficient 0.83 was used (normalized
298 *FLC1/FLC2* expression = 0.83 x Ct_{*TUB6*} - Ct_{*FLC1/FLC2*}).

299

300 *Sequence analysis*

301 To study the *FLC1* DNA sequence in the parental populations and to compare the sequence with
302 that of *FLC2*, we sequenced the promoter and gene sequences of both *FLC* genes of two randomly
303 chosen Sp and Pl individuals [*FLC1*: from -2894 to +5998 (sequence set 1); *FLC2*: from -832 to
304 +6071 (sequence set 2); site information based on the published *A. lyrata* sequence (Hu *et al.*, 2011)
305 and indicated from the start of the transcription start; for some individuals the obtained sequence
306 was slightly shorter]. Due to technical issues, we did not obtain the full gene sequence of *FLC2* for
307 both Pl individuals, and the Pl sequence was obtained by combining the sequences of the two
308 individuals (individual Pl56: from -809 to +3707 and from +3809 to +6071; individual Pl59: from
309 +3708 to +3808). To further study the *FLC1* sequence in the parental populations of the QTL
310 mapping experiment, we also sequenced the full gene region of one of each F2-homozygote for
311 parental alleles (genotype classes Pl₁Pl₂ and Sp₁Sp₂; sequence set 3). Note that these parental alleles
312 represent two random alleles from each natural population. Finally, we studied the *FLC1* sequence
313 polymorphism by sequencing a shorter region of 3529 bp, including 1134 bp from the end of the
314 promoter, the first exon and 2210 bp from the beginning of intron 1 (from -1134 to +2395 bp) in 13
315 randomly chosen Pl and 7 Sp individuals (sequence set 4). Preliminary data indicated that the main
316 differences between populations are found in this part.

317

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

324

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

330
331 We calculated the divergence between *A. lyrata FLC1* and *FLC2* at nonsynonymous (K_A) and
332 synonymous (K_S) sites and their ratio (K_A/K_S ; Nei & Gojobori, 1986; sequence sets 1 and 2). In
333 addition, divergence between *FLC1* and *FLC2* from the *A. thaliana FLC* locus was studied
334 separately. Some PI individuals were excluded prior to analysis due to missing data. Before the
335 analyses, the PI and Sp sequences were pooled for each gene separately to gain one *A. lyrata*
336 sequence for both genes. The level of polymorphism in *FLC1* within Sp and PI populations was
337 quantified separately using the whole *FLC1* gene sequences (sequence sets 1 and 3) and using the
338 shorter sequences (sequence set 4) as the number of segregating sites (S) and as the average number
339 of pairwise differences at silent sites (π ; Tajima, 1983). The divergence between PI and Sp
340 populations was quantified, using the same sequence sets, as the average number of nucleotide
341 substitutions per site between populations (D_{xy} ; Nei, 1987), as F_{ST} (a measure of population
342 differentiation based on mean pairwise differences; Hudson *et al.*, 1992), and as the number of fixed
343 differences between populations. Summary statistics for sequence data were calculated using
344 DnaSP v. 5.0 (Librado & Rozas, 2009).

345

346 **Results**

347 *Effects of vernalization on flowering*

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

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

356

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

365 *FLC1 and FLC2 comparison*

366 Comparing the sequence of *FLC1* to that of *FLC2* (sequence sets 1 and 2) showed that the genes
367 show high similarity from about 350 bp before the first exon to the end of the gene. Upstream of
368 this, the genes were so different that they could not be aligned. The exons of the genes were very
369 similar: there were nine synonymous and seven nonsynonymous differences between *FLC1* and
370 *FLC2* (Fig. 3b). Most of the nonsynonymous differences were detected in exon 4, whereas the
371 synonymous changes were distributed more equally between the exons (Fig. 3b). An insertion of
372 1651 bp, introducing stop codons, was detected in exon 4 of *FLC2* in Spiterstulen (Fig. 3a,b). Intron
373 1 of *FLC1* was 880-891 bp shorter and intron 6 was 576-590 bp longer than those of *FLC2* (Fig. 3a),
374 depending on the population. The divergences for nonsynonymous (K_A) and synonymous sites (K_S)

375 between *FLC1* and *FLC2* were 0.016 and 0.073, respectively. A comparison of *FLC1* and *FLC2*
376 with *A. thaliana FLC* showed that the divergence in these genes were quite similar based both on
377 nonsynonymous and synonymous sites (K_A for *FLC1* = 0.034 and for *FLC2* = 0.028; K_S for *FLC1* =
378 0.120 and for *FLC2* = 0.115) and on K_A/K_S ratio (for *FLC1* = 0.283 and for *FLC2* = 0.243). The
379 insertion in Sp *FLC2* was excluded from the sequence prior to the analysis.

380

381 The *FLC1* and *FLC2* expression comparison showed that both *FLC* genes were expressed in the
382 populations, but they showed different expression patterns among the F2 genotype classes (Fig.
383 4a,b). Expression of *FLC1* was highest in Sp homozygotes (mean value for normalized *FLC1*
384 expression: -6.67) and lowest in Pl homozygotes (-9.24; analysis of covariance, $df = 1$, $F = 156.30$,
385 $P < 0.001$; Fig. 4a). Interestingly, the likely nonfunctional Sp alleles of *FLC2* were also expressed,
386 but the Sp homozygotes showed the lowest expression. In the other genotype classes, *FLC2* was
387 expressed at a higher and fairly similar level (Fig. 4b).

388

389 *Effects of rosette vernalization and population on FLC1 expression*

390 The overall effect of rosette vernalization was to decrease *FLC1* expression (analysis of covariance,
391 $df = 1$, $F = 28.27$, $P < 0.001$, Fig. 5). Before vernalization plants from the northern Sp population
392 had a tendency to show highest *FLC1* expression (mean value for normalized *FLC1* expression: -
393 12.15) and plants from the southern Pl population the lowest (-13.06). After taking into account the
394 effect of vernalization, the between-population difference in *FLC1* expression was close to
395 significant (analysis of covariance, $df = 2$, $F = 2.94$, $P = 0.061$). When *FLC1* expression was
396 analyzed separately within non-vernalized or vernalized plants, significant differences between
397 populations were not detected in these small samples ($df = 2$, $F = 2.43$, $P = 0.109$ and $df = 2$, $F =$
398 1.00, $P = 0.380$, respectively).

399

400 *QTL mapping of FLC1 expression*

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

410

411 *FLC1 sequence analysis*

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

419

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

424 mostly (Fig. 7a). Overall, F_{ST} between the populations was very high (0.62 for the whole gene and
425 0.85 for the shorter region; Table 4).

426

427 Discussion

428 *Vernalization can have multiple effects in A. lyrata*

429 Vernalization is known to have many effects on flowering in different taxa. For instance,
430 vernalization has been shown to decrease heading/flowering time in *Phleum pratense* (Fiil *et al.*,
431 2011), in *A. thaliana* (Shindo *et al.*, 2006) and in *A. lyrata* (Kuittinen *et al.*, 2008) as well as
432 flowering probability in *Bromus tectorum* (Meyer *et al.*, 2004). Here we showed that like in many
433 perennials, adult *A. lyrata* plants responded more to vernalization than juvenile ones. Similar results
434 were found when flowering responses were studied in three *A. lyrata* populations after seed
435 vernalization and after overwintering as rosettes (Riihimäki & Savolainen, 2004), as natural winter
436 increased the flowering probability more than seed vernalization. In contrast, vernalization given at
437 seed stage is more effective than at rosette stage in *A. thaliana* (Nordborg & Bergelson, 1999) and
438 seed and rosette vernalization were shown to have similar effects on flowering in the annual grass *B.*
439 *tectorum* (Meyer *et al.*, 2004). Annual plants can invest most of the resources into flowering and
440 reproduction, but for perennial plants it is important to flower after gaining enough resources for
441 flowering as well as for forthcoming growing seasons. Vernalization also synchronizes flowering of
442 the individuals of the same population, as, in this study, variation in flowering time was greatly
443 reduced after rosette vernalization.

444

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

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

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

458
459 Based on our results the two *FLC* genes in *A. lyrata* have different expression patterns. In the F2
460 plants, the Sp alleles showed higher *FLC1* expression than PI alleles, but for *FLC2* the results were
461 opposite. If flowering time and vernalization requirement was regulated by an *FLC* gene in *A.*
462 *lyrata*, it would be expected to have higher expression in the northern Sp population due to its
463 stronger vernalization requirement compared to the more southern PI population. Based on this
464 reasoning, our results indicate that *FLC1* has the main role in governing the vernalization
465 requirement for flowering in *A. lyrata*.

466
467 In addition to the vernalization response, *FLC* also has other functions in *A. thaliana*: it has been
468 shown to affect flowering via the ambient temperature pathway, to be involved in the control of the
469 circadian clock (reviewed by D'Aloia *et al.*, 2008) and of seed germination (Chiang *et al.*, 2009)
470 and in many other developmental pathways (Scarcelli *et al.*, 2007; Deng *et al.*, 2011). It is thus
471 possible that both *FLC* genes are functional at least in some populations of *A. lyrata*, but they are
472 involved in different developmental pathways. Both genes may still share the same function, but
473 they may be expressed in different tissues or their expression may be reduced compared to the

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

486
487 *Flowering time differences between A. lyrata populations may be affected by differences in FLC1*
488 *expression*

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

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

509

510 In addition to *FLC*, there are also other vernalization-responsive genes in *A. thaliana* (Michaels &
511 Amasino, 2001) that may be involved in the differentiation of the vernalization responses in natural
512 populations. These include *VERNALIZATION INSENSITIVE 3* (Sung & Amasino, 2004),
513 *VERNALIZATION 1* (Levy *et al.*, 2002) and *VERNALIZATION 2* (Gendall *et al.*, 2001). Flowering
514 time variation in *A. thaliana* is mainly explained by several large-effect loci (e.g. Salomé *et al.*,
515 2011), for instance *FLC*, *FRI* and *MADS AFFECTING FLOWERING (MAF)* genes. It was
516 previously shown that *FRI* and autonomous pathway genes balance each other to gain the
517 appropriate level of *FLC* expression (Choi *et al.*, 2011). Here we found no indication that any
518 upstream genes would be involved in population differentiation in *FLC* expression, since the only
519 QTL was in the *FLC* region itself. However, in the QTL mapping experiment the results depend on
520 the genotypes of the parents of the F2 cross. For example, it was shown earlier that a polymorphism
521 in *FRI* affects flowering time variation within *A. lyrata* populations (Kuittinen *et al.*, 2008).
522 However, this polymorphism did not segregate in the F2 cross we studied. We have also measured

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

525
526 *Signs of directional selection in the regulatory regions of FLC1 in the northern Spiterstulen*
527 *population*

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

532
533 Nucleotide variation was strongly reduced in Sp population and differentiation between Sp and PI
534 populations was increased, consistent with recent directional selection (Smith & Haigh, 1974). The
535 observed F_{ST} (0.62) is much higher than F_{ST} between the PI population and a Swedish Stubbsand
536 population (0.2) based on almost 80 different loci (Ross-Ibarra *et al.*, 2008). The 5.5-fold difference
537 of neutral sequence variation in Sp compared with PI highly exceeded the twofold reduction
538 detected at neutral microsatellite loci (Muller *et al.*, 2008). Moreover, in the promoter region, exon
539 1 and part of intron 1, the populations diverged more compared to other regions of the *FLCI* gene
540 (variation reached 15-fold difference). Further, these regions were also highly divergent compared
541 to the corresponding regions in *A. thaliana* (i.e. they were impossible to align), which can be
542 interpreted as high mutation rate at these regions. Thus, low variation in the promoter region and in
543 the first intron in Sp is in contrast to divergence. Hence, it is possible that these expression
544 regulating regions of *FLCI* have been under directional selection in recent history.

545
546 Previous studies in *A. thaliana* have identified regions important for *FLC* expression in non-
547 vernalized plants particularly in the promoter. Moreover, regions affecting both the *FLC* repression

548 during cold and the maintenance of the repression at normal temperatures have been located both to
549 the promoter and the first intron (Sheldon *et al.*, 2002). For instance, a protein complex including
550 FRI binds to the *FLC* promoter and leads to an active chromatin state and high expression (Choi *et*
551 *al.*, 2011). Furthermore, Choi *et al.* (2011) identified a 15 bp binding site sequence for one of the
552 complex components (SUF1) at -363 to about -331 from the transcription start site of *FLC*. A
553 similar motif (differing by two substitutions) was identified in the promoter sequence of *FLC1* from
554 the PI population. This putative motif is located in the region where the Sp *FLC1* sequence has a
555 350 bp deletion and thus lacks the motif. However, as the motif in *A. thaliana* confers high
556 expression, the expectation would be that the Sp deletion would lead to lower *FLC1* expression,
557 which is not supported by data from this study. Clearly, functional studies of the promoter and
558 intron sequence variation observed in *FLC1* from Sp and PI are needed to clarify their effect on
559 *FLC1* regulation. Population genomic analysis of these regions can also give cues on the
560 importance of the different loci.

561

562 **Acknowledgments**

563 We thank Jarkko Vehkaoja for his help with the vernalization experiments in the greenhouse. The
564 undergraduate students in the Savolainen laboratory and lab technicians both in the Savolainen and
565 in the Lagercrantz laboratory are acknowledged for their help with the lab work. We thank Tanja
566 Pyhäjärvi for her contribution to the sequence analysis and Esa Aalto, Tiina Mattila and Antti
567 Virtanen for their advice with CodonCodeAligner. We also thank the members of the Plant
568 Genetics Research Group for their valuable comments on the early versions of the manuscript. The
569 research was supported by the Finnish Population Genetics Graduate School, Biocenter Oulu, ERA-
570 net Plant Genomics and the Swedish Research Council. Oulun Luonnonystävät is acknowledged for
571 a travel grant for Ulla Kemi.

572

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756 **Figure legends**

757 **Fig. 1** Effects of rosette (left) and seedling vernalization (right) on (a) days to flowering, (b) leaf

758 number at flowering start, (c) rosette size at flowering start, and (d) flowering probability, in

759 German Plech (Pl), Norwegian Spiterstulen (Sp) and Swedish Mjällom (Mj) *Arabidopsis lyrata*

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

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

770

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

779

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

785 $Ct_{FLC1/FLC2}$). The median is indicated by thick line, lower and upper quartiles by boxes and outliers
786 by dots. The significance of *FLC1* expression difference between Pl and Sp homozygotes was
787 analyzed with analysis of covariance.

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

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

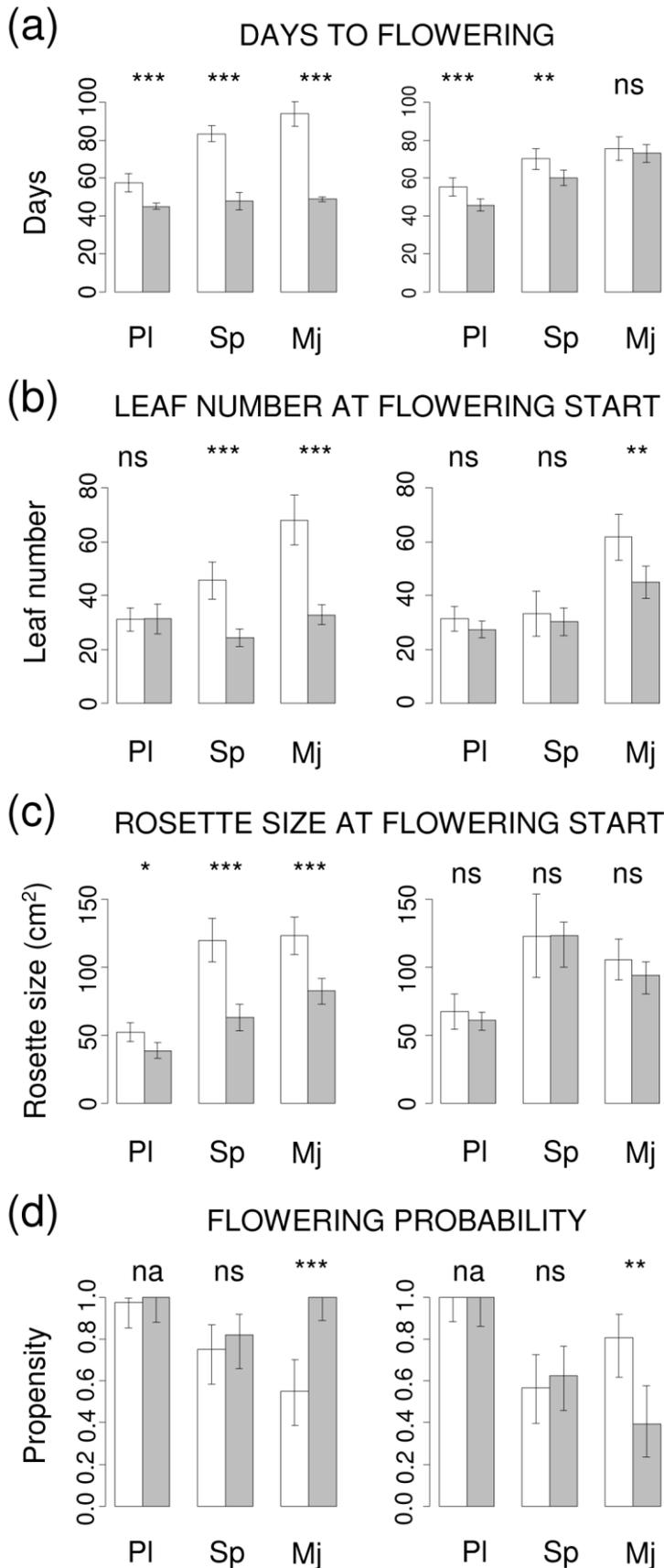
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802 **Fig. 7** (a) Divergence, measured as Dxy, between Spiterstulen and Plech populations of *Arabidopsis*
803 *lyrata* in *FLOWERING LOCUS C 1 (FLC1)*. (b) Sliding window for silent nucleotide diversity (π)
804 across *FLC1* in *Arabidopsis lyrata*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

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Fig. 1

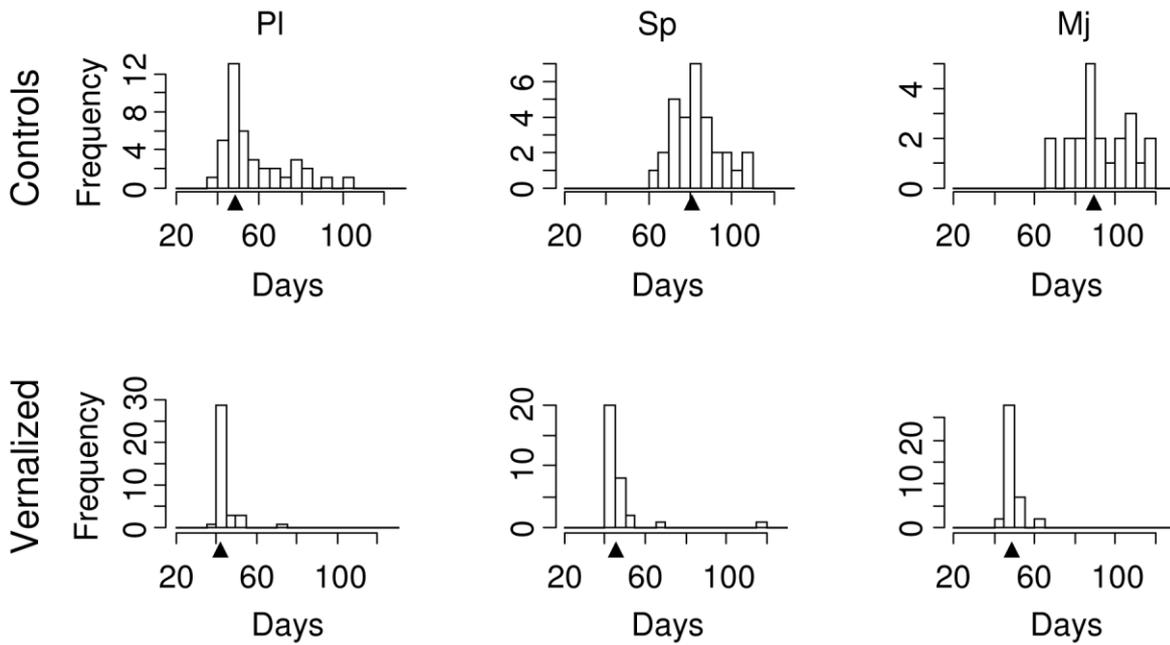
Rosette vernalization Seedling vernalization



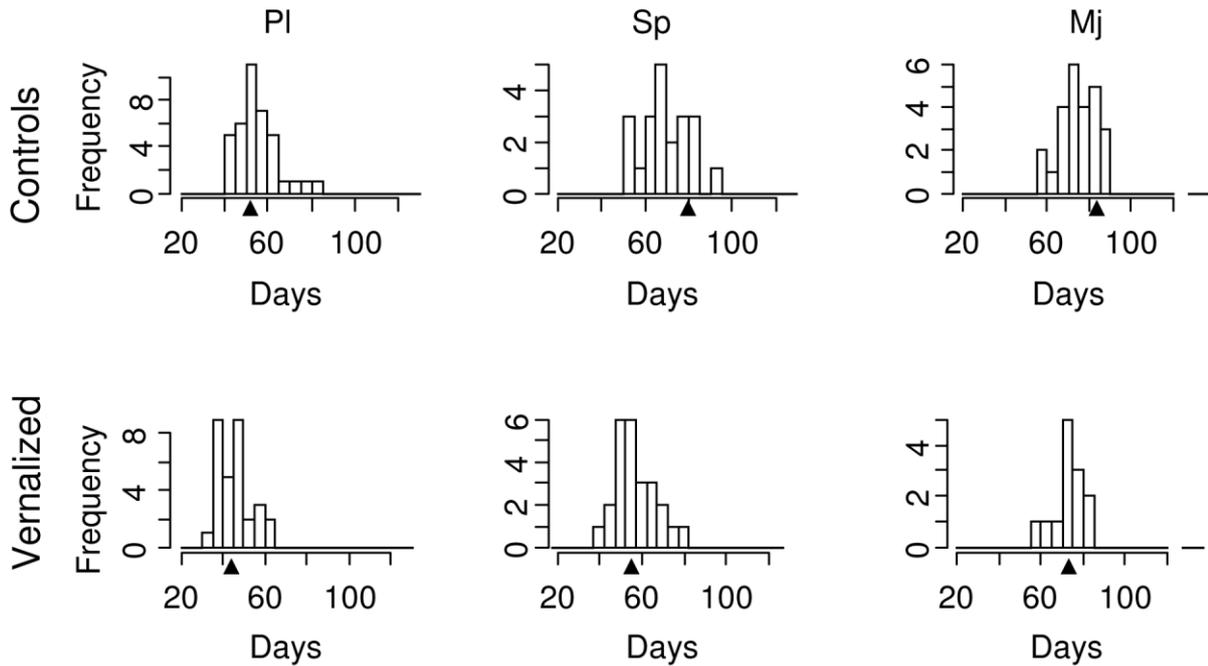
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832 Fig. 2

(a) Rosette vernalization



(b) Seedling vernalization



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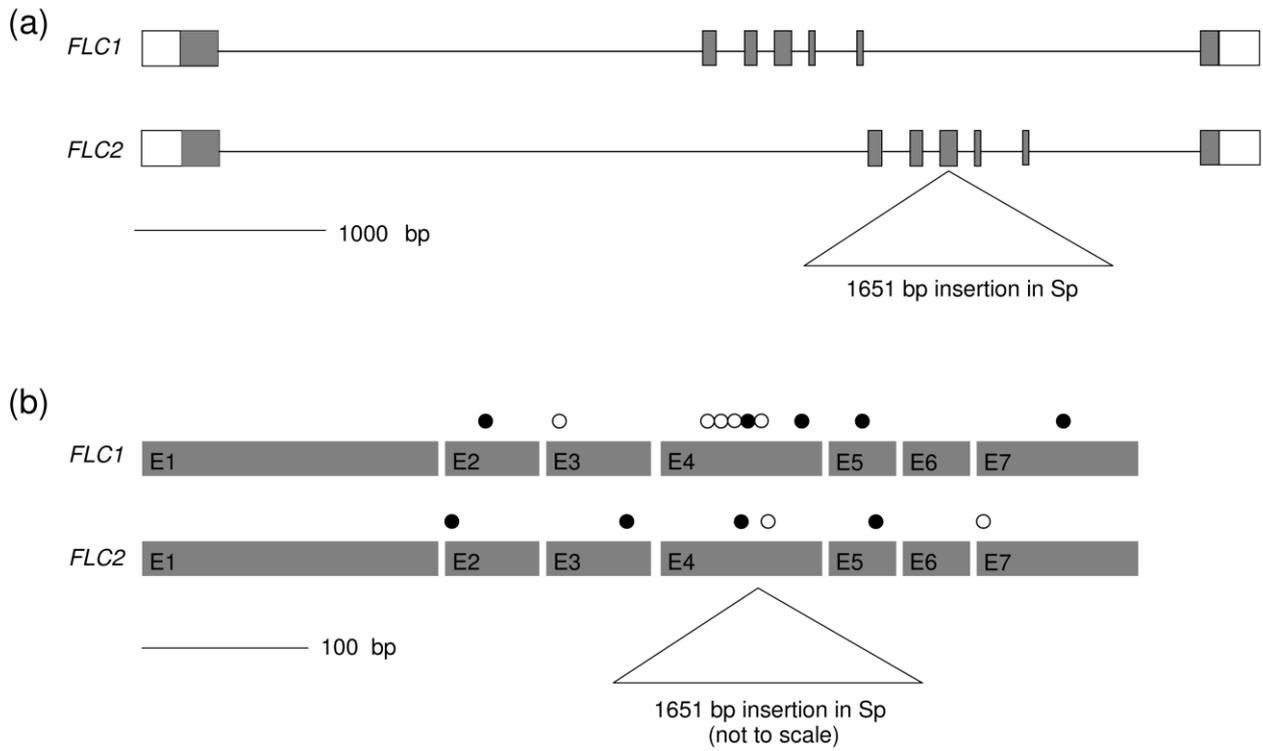
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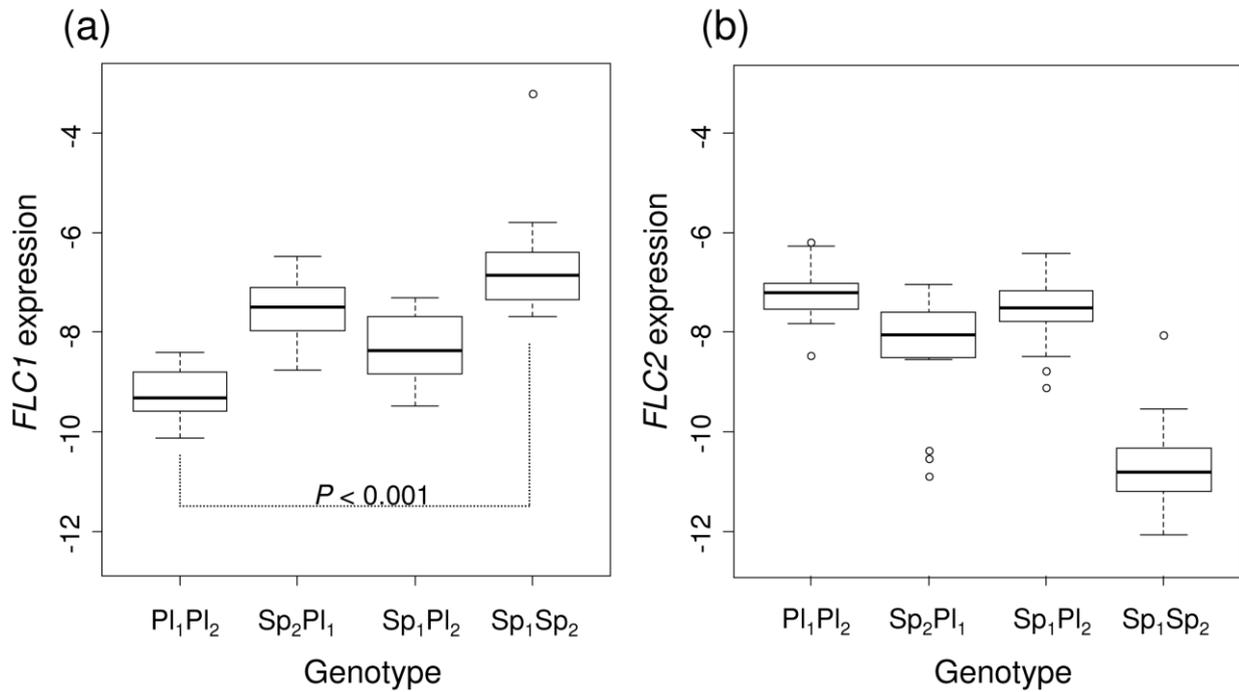
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Fig. 3



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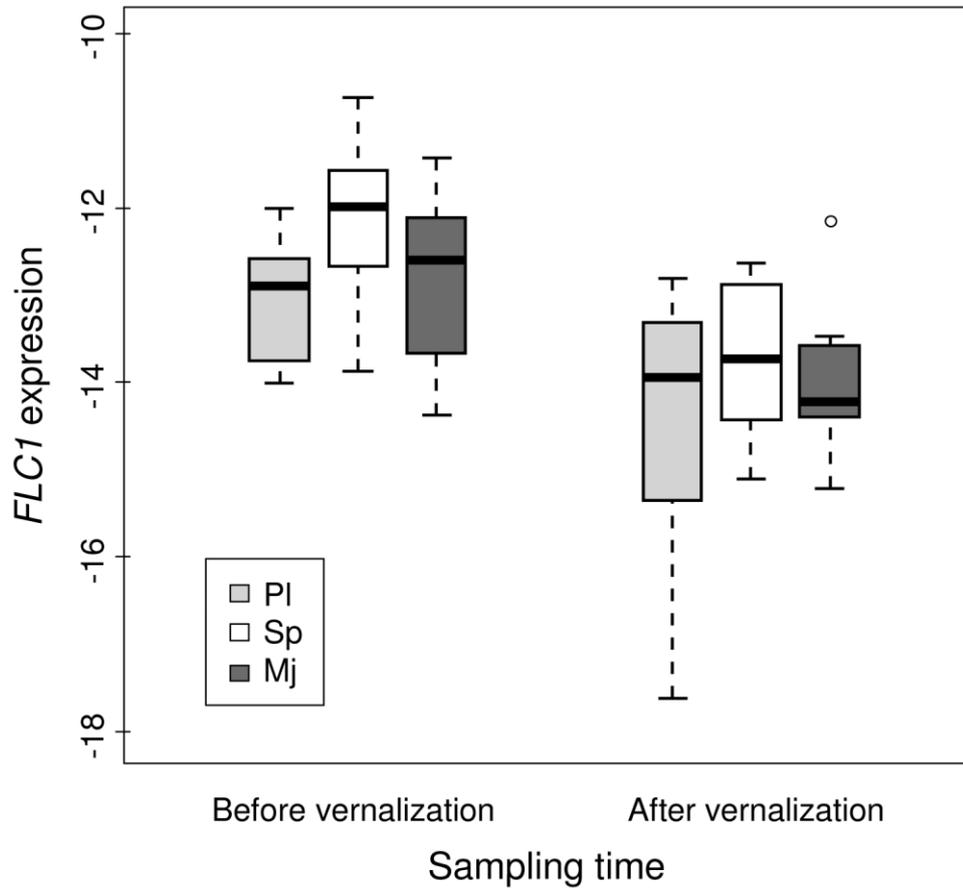


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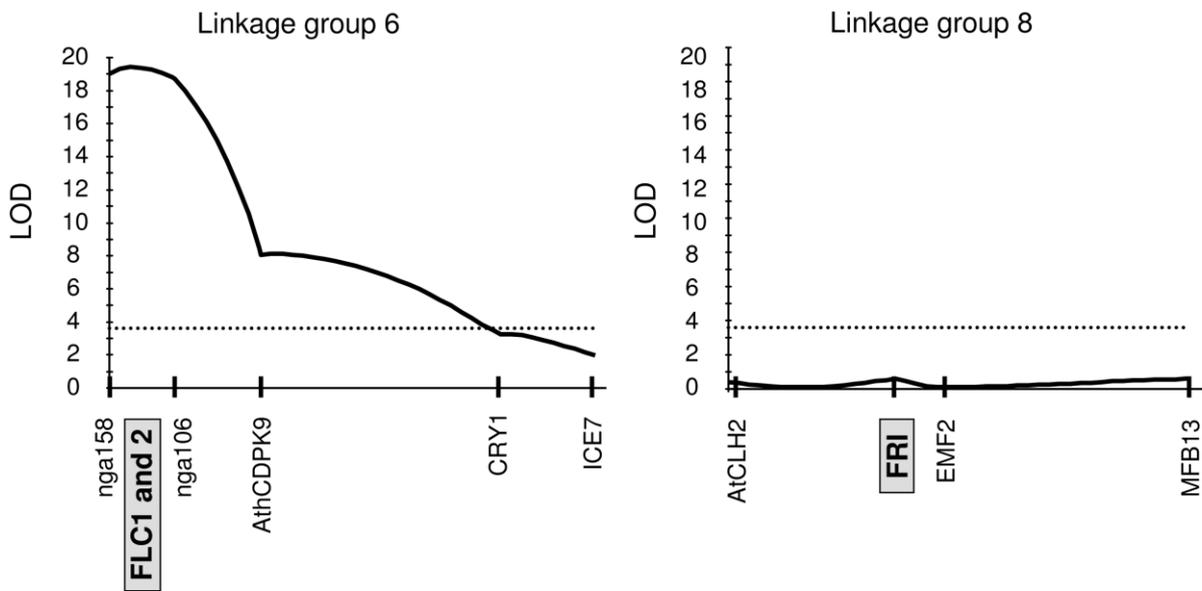
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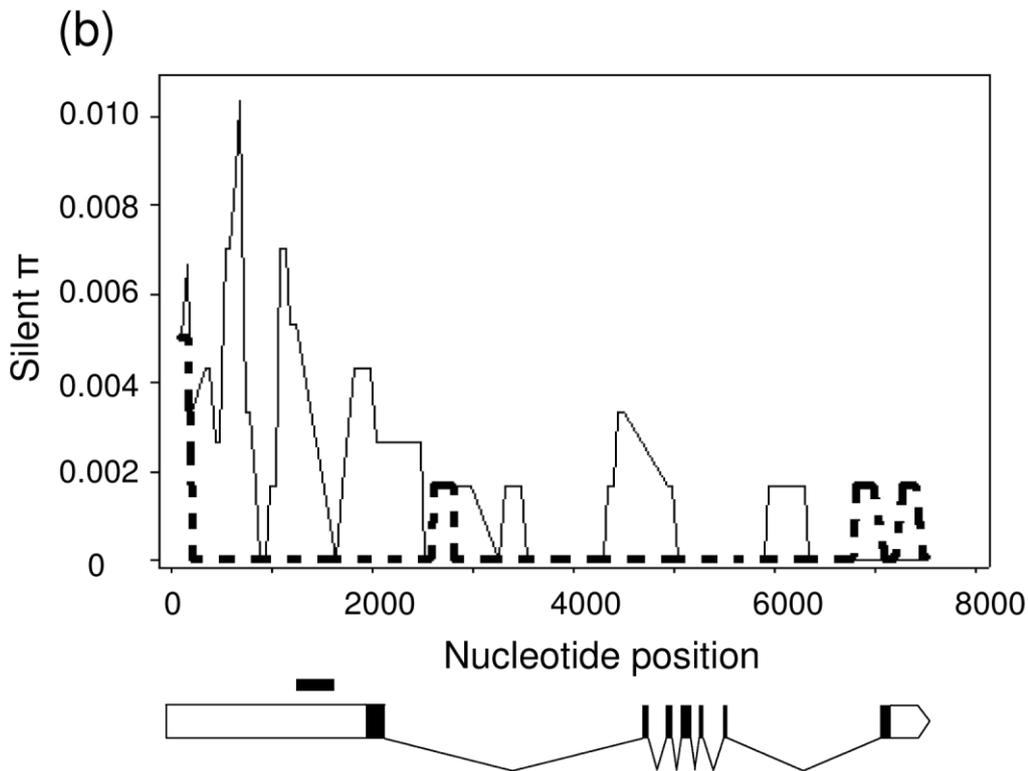
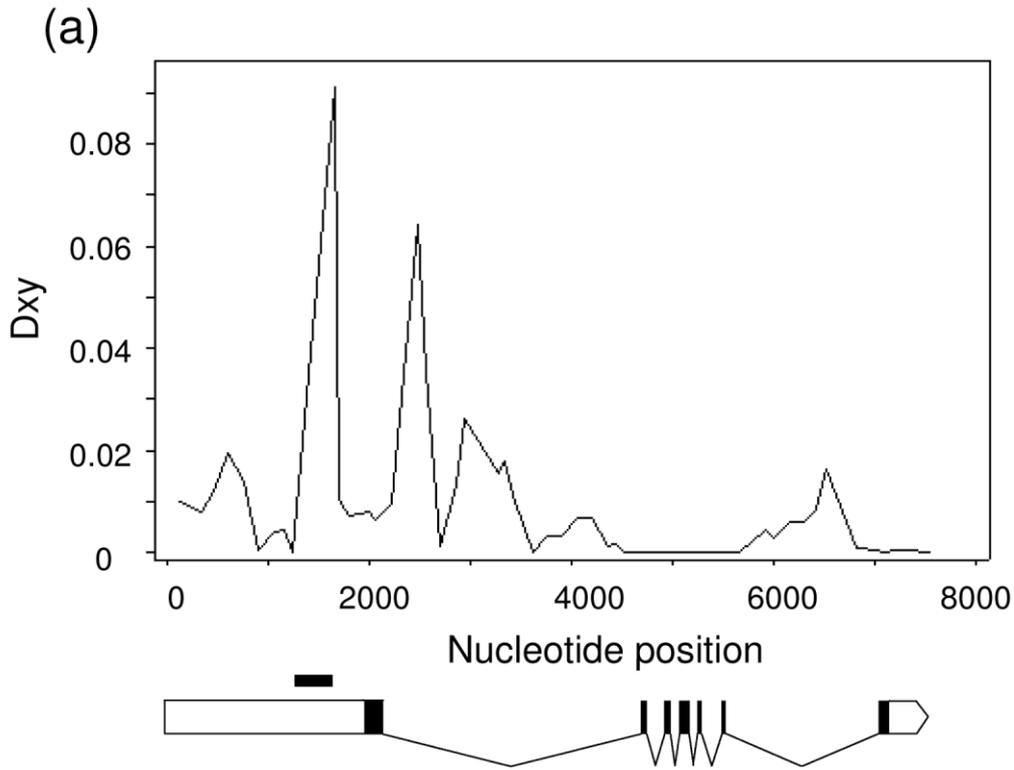
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Kemi, U. . (Auteur de correspondance), Niittyvuopio, A. ., Toivainen, T. ., Pasanen, A. ., Quilot-Turion, B., Holm, K. ., Lagercrantz, U. ., Savolainen, O. ., Kuittinen, H. (2013). Role of vernalization and of duplicated FLOWERING LOCUS C in the perennial *Arabidopsis lyrata* . *New Phytologist*, 197 (1), 323-335. DOI : 10.1111/j.1469-8137.2012.04378.x



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

Experiment	Gene	Forward primer	Reverse primer
<i>FLC1</i> pop, QTL	<i>FLC1</i>	CATGAGCTACTTGAACCTTGTGGAAA	TTCGGCACTCACATTATTGACAT
<i>FLC</i> comp	<i>FLC1</i>	GAGTGCCGAAACTCTTCTTCAACTA	GCCAAAACCTGGTTCTCTTCT
<i>FLC</i> comp	<i>FLC2</i>	AGCAAGCTTGTGGGATCAAA	CCTCCAATTGAACAAGAGTATCGA
<i>FLC1</i> pop, QTL, <i>FLC</i> comp	<i>TUB6</i>	ACCACTCCTAGCTTTGGTGATCTG	AGGTTCACTGCGAGCTTCCTCA

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

Gene	Forward primer	Reverse primer
<i>FLC1</i>	TGAGTCAGGAACGAGTCACG	CTTGTCGGCTACTTTTGT
	CATTTACCAAAGAAAGGTAATGC	TGTAAACGCAGCCTCAATCTT
	CCCATGTCATCACTTTGTGG	TCCAACCATTTCGCGTTTATT
<i>FLC1</i> shorter	GTTAGCTTTCGCCAGTTTG	TGTAAACGCAGCCTCAATCTT
<i>FLC2</i>	AACGAGCAAATGAATGCAAA	AATATATTGGAGGGTTGTAGTAACA
	AATCTTCAGTTTTGTGCTCTTACTT	CACACTGTCTAACCCCGAGT
	TGGGAAGTCTAGGCTTTGGTT	GCCAAAACCTGGTTCTCTTCT
	TTTGCAACTACTTCCAATGC	CAAACGCTCGCCCTTATCA

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895 **Table 3** Effect of vernalization, *Arabidopsis lyrata* population and their interaction on traits of
896 interest in rosette and seedling vernalization experiments, from the likelihood ratio tests between
897 linear models. a) Log₁₀ transformation.

	Vernalization x population			Vernalization			Population		
	df	F	P	df	F	P	df	F	P
Rosette vernalization									
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									
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

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

Population	Sequence			Polymorphism within populations		Divergence between populations (e)		
	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			