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1 # Altered regulation of *TERMINAL FLOWER 1* causes the unique
2 # vernalisation response in an arctic woodland strawberry accession

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Discussion	1512		
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18 Brief heading: Vernalisation response in an arctic woodland strawberry accession

19

20 ## Summary

21 • Vernalisation requirement is an agriculturally important trait that postpones the development
22 of cold-sensitive floral organs until the spring. The Rosaceae family includes many
23 agriculturally important fruit and berry crops that suffer from crop losses caused by frost
24 injury to overwintering flower buds. Recently, a vernalisation-requiring accession of the
25 Rosaceae model woodland strawberry (*Fragaria vesca* L.) has been identified in northern
26 Norway. Understanding the molecular basis of the vernalisation requirement in this
27 accession would advance the development of strawberry cultivars better adapted to
28 temperate climate.

29 • We use gene silencing, gene expression analysis, genetic mapping, and population genomics
30 to study the genetic basis of the vernalisation requirement in woodland strawberry.

31 • Our results indicate that the woodland strawberry vernalisation requirement is endemic to
32 northern Norwegian population, and mapping data suggests the orthologue of *TERMINAL*
33 *FLOWER1* (*FvTFL1*) as the causal floral repressor. We demonstrate that exceptionally low
34 temperatures are needed to down-regulate *FvTFL1* and to make these plants competent to
35 induce flowering at cool post-vernalisation temperatures in the spring.

36 • We show that altered regulation of *FvTFL1* in the northern Norwegian woodland strawberry
37 accession postpones flower induction until the spring, allowing plants to avoid winter
38 injuries of flower buds that commonly occur in temperate regions.

39 Keywords: devernalisation, flowering, temperature, *TERMINAL FLOWER1*, vernalisation,
40 woodland strawberry

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46 ## Introduction

47 Plants use light and temperature cues to adjust their growth and development to particular times of
48 the year. This is especially important for temperate zone perennial species that must be able to
49 survive and reproduce at the same location for several years. Many temperate zone plants, including
50 most strawberry accessions, are short-day (SD) plants, which are induced to flower during the SDs
51 of autumn, and normally flower the following spring (reviewed in Kurokura *et al*., 2013). Some
52 plants have adapted to the temperate climate by developing a requirement for a prolonged period of
53 cold before becoming competent to receive other flower-inducing signals. This process is termed
54 vernalisation (Chouard, 1960), and it has been described at the molecular level in species as diverse
55 as *Arabidopsis thaliana* (Song *et al*., 2012), the temperate grasses *Hordeum vulgare* and *Triticum*
56 *aestivum* (Trevaskis *et al*., 2007), and *Beta vulgaris* (Pin *et al*., 2012).

57 Molecular studies of the vernalisation response in these species have revealed species-specific
58 repressors which must be silenced before flower induction can occur. The vernalisation pathway has
59 been most extensively studied in winter-annual *Arabidopsis*, in which a MADS box transcription
60 factor FLOWERING LOCUS C (FLC) plays a central role in the repression of flowering prior to
61 vernalisation (Michaels & Amasino, 1999) and is up-regulated by FRIGIDA (FRI) in non-
62 vernalised plants (Johanson *et al*., 2000; Michaels & Amasino, 2001). FLC delays flowering by
63 binding to the regulatory regions of several genes encoding floral activators; *FLOWERING LOCUS*
64 *T* (*FT*) in the leaves and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FD*
65 in the shoot apical meristem (SAM) (Helliwell *et al*., 2006; Searle *et al*., 2006). Upon vernalisation,
66 the *FLC* locus is trimethylated at lysine 27 of histone 3 by the action of Polycomb repressive
67 complex 2 (PRC2) (Wood *et al*., 2006). After vernalisation, *FLC* remains epigenetically and stably
68 silenced under warm conditions (Michaels & Amasino, 1999), enabling the long day (LD)
69 dependent upregulation of *FT* and *SOC1*. In the SAM, the FT protein forms a heterodimer with FD,
70 which promotes flowering by activating the meristem identity gene *APETALA 1* (*AP1*) (Abe *et al*.,
71 2005; Wigge *et al*., 2005).

72 In a closely related perennial species *Arabis alpina*, an FLC orthologue PERPETUAL
73 FLOWERING 1 (PEP1) causes a vernalisation requirement (Wang *et al*., 2009). PEP1 also inhibits
74 flowering by repressing the *A. alpina* orthologue of *SOC1* (Wang *et al*, 2011). However, in *A.*
75 *alpina*, silencing of *PEP1* is only transient, enabling the plant to undergo repeated cycles of

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76 flowering and vegetative growth typical of the life cycle of perennial plants. The vernalisation
77 response in *A. alpina* also depends on the age of the plants; only plants more than five weeks old
78 show a full flowering response to vernalisation (Wang *et al*., 2011).

79 In temperate grasses, the requirement for vernalisation is caused by epistatic interactions between
80 three loci, *VRN1 (*Danyluk *et al*., 2003; Yan *et al*., 2003; Trevaskis *et al*., 2007), *VRN2* (Yan *et al*.,
81 2004) and *VRN3* (Yan *et al*., 2006). *VRN1* is the grass orthologue of the floral meristem identity
82 gene *AP1* (Yan *et al*., 2003), while *VRN3* encodes the grass orthologue of FT (Yan *et al*., 2006).
83 VRN2 is a CCT domain protein that does not have close homologues in *Arabidopsis*, but plays a
84 similar role to FLC; it is a dominant flowering repressor and is down-regulated by vernalisation and
85 SDs (Yan *et al*., 2004). Similarly to *FT*, *VRN3* is expressed in leaves under LD conditions in both
86 wheat and barley (Yan *et al*., 2006), and in wheat, VRN3 forms a complex with the wheat
87 orthologue of FD (TaFDL2) to activate *VRN1* (Li *et al*., 2008). However, in wheat, the three genes
88 form a regulatory feedback loop not characterized in *Arabidopsis*; loss of *VRN2* results in elevated
89 levels of *VRN3* and *VRN1* transcripts and promotes flowering, and the up-regulation of *VRN1*
90 further down-regulates *VRN2*. Also the spatial regulation of the *AP1* orthologue expression is
91 divergent; in *Arabidopsis*, *AP1* is expressed almost exclusively in flower meristems, whereas in
92 wheat, *VRN1* is expressed also in leaves (Yan *et al*., 2003).

93 In cultivated beet (*Beta vulgaris*), both vernalisation-requiring biennial forms and annual forms,
94 which flower in LDs without vernalisation, have been characterised. The interactions of *BOLTING*
95 *TIME CONTROL 1* (*BvBTC1*), and two homologues of *FT* (*BvFT1* and *BvFT2*), determine the
96 vernalisation response. *BvBTC1* encodes a pseudo-response regulator with homology to circadian
97 clock genes in *Arabidopsis* (Pin *et al*., 2012). BvFT2 is the functional orthologue of FT and
98 promotes flowering, whereas BvFT1 has evolved into a floral repressor (Pin *et al*., 2010). In annual
99 beet, *BvBTC1* is up-regulated by LDs, leading to repression of *BvFT1* and up-regulation of the
100 floral promoter *BvFT2*. In biennials, *BvBTC1* does not respond to LDs due to a large insertion in the
101 promoter region, and a prolonged period of cold is required to increase *BvBTC1* transcript levels
102 sufficiently to down-regulate *BvFT1* and up-regulate *BvFT2* (Pin *et al*., 2012).

103 Heide and Sønsteby (2007) identified an obligatory vernalisation requirement in a diploid woodland
104 strawberry (*Fragaria vesca* L.) accession from Northern Norway (referred to hereafter as NOR1), a
105 phenomenon that has previously not been reported in the *Fragaria* genus or characterised in the

106 Rosaceae family. Typically, both woodland strawberries and the octoploid cultivated strawberries
107 (*F.* × *ananassa* Duch.) are induced to flower by SDs and/or cool temperatures (Darrow & Waldo,
108 1934; Guttridge, 1985; Heide *et al*., 2013) which down-regulate *TFL1* homologues encoding key
109 floral repressors (Koskela *et al*., 2012; Rantanen *et al*., 2015; Koskela *et al*., 2016). In contrast to
110 SD genotypes, NOR1 does not flower after an exposure to SDs at 9°C, whereas field-grown plants
111 flower after overwintering albeit considerably later than other Norwegian accessions. Furthermore,
112 under a controlled climate, 38% of plants flowered after a 15-week vernalisation period at 2°C,
113 indicating that NOR1 has special environmental requirements for flowering (Heide & Sønsteby,
114 2007).

115 Here, we describe a novel role for *FvTFL1* in the vernalisation response of NOR1. We show that the
116 regulation of *FvTFL1* differs between NOR1 and FIN56 (the SD *F. vesca* accession PI551792)
117 under a range of conditions, and demonstrate that NOR1 requires exceptionally low temperatures to
118 fulfill the vernalisation requirement, followed by cool temperatures to induce flowering.
119 Furthermore, we provide genetic and functional evidence that *FvTFL1* is needed for the
120 vernalisation response that is unique to the NOR1 accession and has arisen locally in the arctic
121 environment.

122 Materials and methods

123 Plant material

124 The physiology and the genetic basis of the vernalisation response was studied in a previously
125 reported woodland strawberry accession NOR1 originating from Alta, Northern Norway (Heide &
126 Sønsteby, 2007), and in 16 new clones that were collected from the same population (NOR-P1). A
127 Finnish accession FIN56 (PI551792, National Clonal Germplasm Repository, Corvallis, USA) was
128 used as a control. In addition, 78 accessions originating from Finland, Norway and Iceland (Table
129 S1) were used to explore the population structure of the Nordic woodland strawberries. Plants were
130 propagated from runner cuttings and grown in a glasshouse under long day conditions (18h/18°C in
131 Finland, 24h/20°C in Norway) until the beginning of the experiments. In Finland, plants were
132 illuminated using high-pressure sodium lamps (Airam 400W, Kerava, Finland) at 120 µmol $m^{-2}s^{-1}$,
133 and in Norway, 15 µmol $m^{-2}s^{-1}$ incandescent light was applied continuously during plant
134 propagation.

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135 **Field experiments**

136 The effect of vernalisation on flowering and gene expression of NOR1 and FIN56 was tested in
137 field experiments in Helsinki, Finland (coordinates 60° 11' N, 24° 56' E) and at Kapp, Norway (60°
138 40' N, 10° 52' E). Flowering time (the date of the first open flower) was observed either in the field
139 in the spring or after transfer of plants to a glasshouse during winter at the time points indicated in
140 figure legends.

141 To test whether other Nordic woodland strawberry accessions require vernalisation, a total of 67
142 accessions including NOR1 and FIN56 (Table S1) were tested for flower induction in the field in
143 Helsinki during autumn 2014. Four plants per accession were propagated outdoors from runner
144 cuttings at the end of July. Plants were grown outdoors until the 6th of October to give them a
145 natural SD and cool temperature treatment, but no vernalisation (weather conditions Fig. S1), and
146 subsequently, flowering was observed in a glasshouse in LDs at 18°C.

147 **Experiments in controlled climate**

148 The effects of SDs, and vernalisation and post-vernalisation temperature conditions on flowering
149 and gene expression were studied in growth chambers in Helsinki and in a phytotron in Ås, Norway
150 (59° 40'N, 10° 47' E). In SD treatments, in Helsinki, plants were exposed to 12-h SDs at 11°C for
151 periods indicated in figure legends followed by flowering time observations in a glasshouse in LDs
152 at 20°C. To test the vernalisation responses, plants were exposed to temperatures of 0°C, fluctuating
153 -2/+2°C (night/day) or 4°C in SDs for up to 15 weeks in Helsinki or Ås as detailed in figure
154 legends. To test the effect of post-vernalisation temperature conditions, plants were first vernalised
155 in the field at Kapp or growth chamber in Helsinki followed by 5-week treatments at 10°C and
156 22°C in LDs in growth chambers. After vernalisation or post-vernalisation treatments, the date of
157 first open flower was observed in a glasshouse in LDs at 20°C.

158 **Scanning electron microscopy of shoot apices**

159 To observe SAM morphology after vernalisation, vegetative NOR1 plants with 5–7 large branch
160 crowns were moved onto field in Helsinki, Finland in June 2016 and kept outdoors until February
161 2017 (weather data, Fig. S2). Following vernalisation, the plants were subjected to LDs at 10°C and
162 shoot apex samples were collected for scanning electron microscopy at time points 0, 1, 2 and 5

163 weeks. On week 5, the remaining plants were moved to LDs at 20°C for flowering observations.

164 Vernalised shoot apex samples were fixed in FAA buffer (50% ethanol, 5% glacial acetic acid, 3.7%
165 formaldehyde) overnight and dehydrated through an ethanol series. Critical point drying was carried
166 out in a Leica EM CPD300 dryer (Leica Mikrosysteme GmbH, Austria) and the samples were
167 examined and imaged using Quanta 250 FEG (FEI, Oregon, USA) scanning electron microscope at
168 the EM Unit of the Institute of Biotechnology, University of Helsinki.

169 RNA extraction and quantitative RT-PCR

170 Samples for RNA extraction were collected at the time points indicated in the figure legends,
171 immediately frozen in liquid nitrogen, and stored at -80°C. RNA was extracted using the pine tree
172 method (Monte & Somerville, 2002), treated with rDNase (Macherey-Nagel GmbH, Düren,
173 Germany) according to manufacturer's recommendations, and cDNA was synthesized from 500 ng
174 total-RNA using Superscript III reverse transcriptase (Invitrogen, Thermo Fisher Scientific, MA,
175 USA). SYBR Green I master mix (Roche Deutschland Holding GmbH) was used for 10 µl real-
176 time PCR reactions and run in a LightCycler 480 instrument (Roche) as described in Koskela *et al*.
177 (2016). Three technical and three biological replicates were performed using the primers listed in
178 Table S2. Relative expression levels were calculated by the $^{\Delta\Delta}$Ct method (Pfaffl, 2001) with *FvMSI1*
179 as a normalisation gene. Log-transformed gene expression data were used for running ANOVA.
180 Results from ANOVA were further subjected to Tukey's HSD or to least-squares means test. All the
181 statistical analyses were done using RStudio version 1.0.136 (RStudio Team, 2016).

182 Generation of crossing populations

183 In order to introduce the *FvTFL1*-RNAi construct into NOR1 background, NOR1 female parents
184 were pollinated with pollen from transgenic *FvTFL1*-RNAi lines in Hawaii-4 (H4) or FIN56 genetic
185 backgrounds (Koskela *et al*., 2012). The transgenic F1 progeny was selected by observing GFP
186 fluorescence in imbibed seeds and grown in a glasshouse in LDs for three months. F2 populations
187 were produced by allowing GFP positive F1 plants to self-pollinate. Transgenic F2 seeds were
188 separated from wild type F2 progeny by observing GFP fluorescence. DNA was extracted from the
189 F2 plants using CTAB (Doyle & Doyle, 1987). LD-grown non-transgenic NOR1 × H4 F2 plants
190 were subjected to six weeks of SDs followed by flowering observations under LD in a glasshouse in
191 Helsinki. The marker *TFL1*-6FAM (Koskela *et al*., 2012) was used for genotyping the NOR1 × H4

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192 F2 population. NOR1 × FIN56 non-transgenic F2 plants were given a SD treatment at 11°C for 8
193 weeks to induce flowering, after which the plants were moved to LDs for flowering observations.
194 The markers listed in Tables S2 and S3 were used to genotype the NOR1 × FIN56 population using
195 capillary electrophoresis (Methods S1), Sequenom MassArray (Agena Biosciences) (Methods S2),
196 and high resolution melting (HRM) analysis (Methods S3). Genetic maps were generated by
197 maximum likelihood mapping algorithm implemented in JOINMAP 4.0 (Kyazma, NL). Default
198 settings were used, except for the initial acceptance probability parameter, which was set to 0.5.

199 Estimation of population structure in woodland strawberry

200 DNA was extracted from 95 genotypes listed in Table S1 with the DNeasy plant mini-kit (Qiagen).
201 Genotyping-by-sequencing (GBS) libraries were produced according to Elshire *et al*. (2011), and
202 libraries were sequenced by the Illumina HiSeq 2000 (Illumina Inc., CA, USA) at the Wiel Medical
203 Centre of Cornell University. The Stacks pipeline, version 1.19, (Catchen *et al*., 2011) was used to
204 call SNPs, and 474,057 SNPs were located to the woodland strawberry reference genome version
205 2.0.a1 (Tennessen *et al*., 2014). Samples were grouped as based on geographic locations: Iceland,
206 the Alta region in Norway, other Norway, Southern, Middle and Northern Finland. Several filtering
207 steps were performed: a SNP was accepted if at least 60% of samples in each group had data at the
208 locus, a SNP was represented by at least six reads and it had a minimum minor allele frequency of
209 0.05. These selection criteria resulted in the further analysis of 7,420 SNPs. Using vcftools
210 (Danecek *et al*., 2011), amplified paralogous loci and genotyping errors were controlled by
211 removing excessively heterozygous SNPs (2pq, $p < 0.05$) and SNPs with high coverage (< 63 =
212 mean depth/SNP (21.5) + 2 sd.) resulting in 2,401 common SNPs in 78 samples. Finally, to avoid
213 linkage disequilibrium between SNPs in population structure analysis, only SNPs located at a
214 minimum distance of 10 kb were selected (1,333 SNPs). Principal component analysis (PCA) was
215 conducted with R (R Core Team 2015) using the SNPrelate package (Zheng *et al*., 2012).

216 Whole genome sequencing and data analysis

217 DNA from NOR1 and FIN56 accessions, and 16 samples from the NOR-P1 population, that were
218 collected at the minimum distance of 10 meters to avoid collecting clones, was extracted using
219 CTAB (Doyle & Doyle, 1987). RNA was excised using RNase (Sigma-Aldrich, Darmstadt,
220 Germany). Whole genome sequencing was carried out using Illumina MiSeq (NOR1 and FIN56) or
221 NextSeq 500 sequencer and SNP/indel calling was carried out as described in Methods S4.

222 To develop markers for Sequenom MassArray genotyping, SNPs carrying different homozygote
223 alleles on linkage group 6 (LG6) in FIN56 and NOR1 were retained, and 27 SNPs with no
224 additional variation within a 200 bp up- or downstream region were selected. Bedtools (Quinlan *et*
225 *al*. 2010) was used to produce fasta files for the development of Sequenom primers (Table S3). To
226 develop additional SNP markers for HRM analysis, LG6 genomic sequences of four NOR1
227 population clones that required vernalisation were compared with 13 individuals that did not require
228 vernalisation to identify completely differentiated SNPs ($F_{ST} = 1$) (Weir & Cockerham, 1984). F_{ST}
229 analysis was also carried out for all the SNPs and indels (size limit 300 bp) detected inside the final
230 mapping window of NOR1 × FIN56 cross.

231 To identify possible structural variations including duplications, insertions and deletions inside the
232 final mapping window the BAM files of population samples were compared using the Integrative
233 Genomics Viewer (IGV, Thorvaldsdóttir *et al*. 2013). Sequencing data is stored at NCBI Short Read
234 Archive (https://www.ncbi.nlm.nih.gov/sra) under the number SRP110929.

235 Results

236 Responses of NOR1 and FIN56 to short days and low temperature

237 To understand the flowering response of the arctic accession NOR1, we compared gene expression
238 patterns of flowering-related genes in NOR1 and FIN56 grown under different environmental
239 treatments. First, we subjected plants to SDs at 11°C for three weeks. No down-regulation of
240 *FvTFL1*, a gene encoding a major floral repressor (Koskela *et al.*, 2012), was observed in shoot
241 apices of NOR1 after the SD treatment compared to LDs, and in SDs, several times higher
242 expression level was observed in NOR1 than in FIN56 (Fig. 1a). At the same time, the up-
243 regulation of the floral marker gene *FvAP1* was observed in FIN56 and these plants flowered about
244 five weeks later (Fig. 1b; Table S4), whereas in NOR1, *FvAP1* expression level remained low and
245 plants stayed vegetative. To understand the role of upstream floral regulators FvFT1 and FvSOC1
246 (Mouhu *et al.*, 2013), we analysed the expression of corresponding genes in leaves and shoot
247 apices, respectively. These genes, however, exhibited similar expression patterns in both accessions
248 and did not correlate with *FvTFL1* mRNA levels in NOR1 (Figs 1c, 1d), indicating that unknown
249 regulator(s) maintain high *FvTFL1* expression level in NOR1 in SDs preventing flower induction.
250 We also studied the expression of another *FT*, a woodland strawberry homolog of *FaFT3* that is

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251 activated in the shoot apices of the cultivated strawberry under flower-inductive SDs (Nakano *et al.*,
252 2015). Similarly to *FvAP1*, *FvFT3* was highly expressed only in FIN56 in SDs (Fig. 1e).

253 Next, we tested the effect of cooler temperatures on NOR1. Fifteen weeks at +4°C did not down-
254 regulate *FvTFL1* (Fig. S3a), and plants remained vegetative after this treatment. Moreover, 2 weeks
255 at ±2°C had no effect on *FvTFL1* expression in NOR1, whereas in FIN56, this treatment down-
256 regulated *FvTFL1* (Fig. 2a), further suggesting that *FvTFL1* regulation is altered in the
257 vernalisation-requiring NOR1. Longer treatment of five or ten weeks at ±2°C or 0°C silenced
258 *FvTFL1* also in NOR1 (Figs 2b, S3b), but *FvSOC1* was significantly down-regulated only after ten
259 weeks (Fig. 2c). Furthermore, *FvAP1* was not up-regulated during ten weeks of vernalisation at
260 ±2°C (Fig. 2d), and only 23% of plants flowered after this treatment indicating that even long
261 period of close-to-zero temperature is not sufficient to induce flowering.

262 Cool post-vernalisation temperature induces flowering in NOR1

263 NOR1 flowered in the field, albeit more than two weeks later than in FIN56 (Figs S4a, S5). These
264 differences were associated with distinct seasonal gene expression patterns. In FIN56, *FvAP1* was
265 already highly activated in October, whereas in NOR1, clear up-regulation was only observed in the
266 spring (Fig. S4b). Our data also indicated that *FvTFL1* was down-regulated earlier in autumn in
267 FIN56 than in NOR1, although these differences were not statistically significant due to high levels
268 of variation (Fig. S4c). Based on these findings, we reasoned that floral initiation in NOR1 might
269 require spring-like conditions, i.e. LDs and cool temperature, after the vernalisation at near-freezing
270 temperatures.

271 To study the effect of post-vernalisation temperature on floral initiation in NOR1, we grew outdoor-
272 vernalised plants (Fig. S6) under controlled climate in LDs at either 10°C or 20°C for five weeks
273 followed by LDs at 20°C. No flowering was observed in plants exposed directly to 20°C after
274 vernalisation, whereas 75% of the plants exposed to 10°C flowered approximately 55 days after the
275 end of vernalisation (Table 1). Similarly, plants exposed to fluctuating temperature between -2°C
276 and +2°C for ten weeks flowered only when they were subsequently grown at 10°C in LDs for five
277 weeks (Table 1). To confirm that flower initiation occurred at 10°C after vernalisation, we observed
278 SAMs of outdoor-vernalised plants using scanning electron microscopy. All the analysed SAMs
279 were in a vegetative state directly after vernalisation (week 0), whilst broader and flatter SAMs

280 were detected already after one week at 10°C (Fig. 3a). After two weeks at 10°C, most SAMs had
281 early-stage flower primordia, and by week 5, inflorescences were visible to the naked eye. We also
282 compared floral development of NOR1 with other individuals of the same NOR-P1 population that
283 did not require vernalisation. In these plants, in contrast to NOR1, flower buds were visible
284 immediately after vernalisation (Fig. S7), confirming that flower initiation occurred in autumn.

285 Next, we explored how the visible changes in SAM morphology correlate with changes in gene
286 expression levels. Corroborating the earlier findings (Hollender *et al.*, 2011; Hollender *et al.*, 2014),
287 *FvAP1* was significantly up-regulated only at 10°C at the same time as the early-stage floral
288 primordia were observed (Figs 3b, S8). Interestingly, *FvFT3* showed significant up-regulation at the
289 same time or slightly earlier than *FvAP1* (Figs 3c, S8), indicating that *FvFT3* may have a role in
290 floral induction. In contrast to these genes, the expression of *FvTFL1* or *FvSOC1* did not correlate
291 with floral initiation and *FvAP1* expression during post-vernalisation temperature treatments (Fig.
292 4). *FvTFL1* was up-regulated after one week at both 10°C and 20°C, and the expression levels
293 remained high throughout the analysed time points (Fig. S8). Taken together, our data support the
294 hypothesis that in NOR1, the down-regulation of *FvTFL1* during winter cold is needed to enable
295 post-vernalisation flower induction and initiation that occurs at cool temperatures in the spring
296 independently of *FvTFL1* mRNA level. This pattern contrasts with the typical seasonal growth
297 cycle of woodland strawberry, in which floral initials are formed during a short period in autumn
298 with flowering taking place the following spring (Heide & Sønsteby, 2007; Koskela *et al*. 2012).

299 **The role of *FvTFL1* in the control of flowering in NOR1**

300 To confirm that the regulation of *FvTFL1* plays a major role in the control of flowering in NOR1,
301 we introduced the *FvTFL1*-RNAi transgene into NOR1 background by crossing NOR1 with
302 previously reported *FvTFL1*-RNAi lines (Koskela *et al.*, 2012). We expected to see flowering in
303 LDs in the NOR1 × FIN56 F1 and F2 plants carrying the *FvTFL1*-RNAi construct. Moreover, if the
304 vernalisation trait was a dominant single-gene trait, we would expect to see SD-induced flowering
305 in approximately 25% of the non-transgenic F2 individuals. Indeed, all the F1 and F2 plants
306 carrying the *FvTFL1*-RNAi construct flowered readily in LDs without vernalisation. Phenotyping
307 the vernalisation requirement in 534 non-transgenic F2 individuals from the NOR1 × FIN56 cross
308 confirmed dominant, single-gene control for the trait (Table S5). Dominant single-gene control was
309 confirmed in another cross using a paternal transgenic line with RNAi-silenced *FvTFL1* in 'Hawaii-
310 4' background (Table S6; Fig. S9; Notes S1).

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311 Using NOR1 × FIN56 F2 population, we mapped the locus causing the vernalisation requirement
312 referred to as '*VERN*' from hereafter (Fig. 5a). The entire population was first genotyped for eight
313 previously published SSR markers (Table S2) to show that *VERN* is located on LG6 of the *F. vesca*
314 Genome v2.0.a1. These initial mapping results placed *VERN* in close proximity to *FvTFL1* and
315 suggested that the order of scaffolds on the reference genome is incorrect (Fig. S10). Therefore, we
316 sequenced the parental genomes and designed 27 SNP markers located on different LG6 scaffolds
317 to be genotyped by Sequenom (Gabriel *et al*., 2009) in 355 F2 individuals. Nineteen markers
318 produced reliable genotypes and 18 of them were mapped onto the LG6 (Fig. S11). However, the
319 mapping window around *VERN* was still 0.7 cM wide.

320 Next, we collected 16 additional clones from the original NOR–P1 population and sequenced their
321 genomes. Since three clones in addition to original NOR1 did not flower after a SD treatment, we
322 searched for high-quality SNPs between non-flowering and flowering individuals and identified 24
323 fully differentiated SNPs and two indels (F_{ST}=1) on LG6 (Table S7). Fifteen out of those SNP/indel
324 markers were polymorphic between NOR1 and FIN56 and could be mapped using HRM
325 genotyping (Li *et al*., 2010) in a set of 93 NOR1 × FIN56 F2 plants that were selected based on the
326 Sequenom genotyping. The resultant genetic map showed that the SNP35-HRM co-segregated with
327 the *VERN* locus in all genotyped F2 individuals, and it was flanked by four markers contained at
328 one end of the scaffold 0513102 (Fig. 5a; Table S8). The mapping of one additional marker, SNP21,
329 confirmed the position of *VERN* inside a 855 kb mapping window (Fvb6: 9814000-10660270) on
330 this scaffold.

331 The polymorphism detected by the SNP35-HRM marker was the only fully differentiated
332 polymorphism (F_{ST} = 1) that was detected inside the mapping window between four vernalisation-
333 requiring and 13 population samples that did not require vernalisation (Fig. 5b). Furthermore,
334 manual inspection of the sequenced genomes did not reveal any additional structural variations in
335 the region. The marker detects a 1 bp deletion (Fig. S12) present in vernalisation-requiring
336 individuals from the NOR–P1 population, located in the putative 5'-promoter of *FvTFL1*, 2,547 bps
337 upstream of the transcription start site, which affects several promoter motifs (Table 2). To confirm
338 that the deletion is associated with altered *FvTFL1* expression, we studied vernalisation-requiring
339 and non-requiring individuals from the NOR–P1 population. Our analysis showed that the level of
340 *FvTFL1* expression was lower in SD-grown non-vernalisation requiring individuals than in
341 individuals with vernalisation requirement (Fig. 6a). Furthermore, *FvAP1* and *FvFT3* were

342 expressed at higher levels in SD-grown non-vernalisation requiring individuals (Figs 6b, 6c).

343 Comparison of FIN56 and NOR1 *FvTFL1* coding sequences did not reveal any differences between
344 the two genotypes (Figure S13). Taken together, our genetic and genomic data indicates that a
345 deletion in the promoter region of *FvTFL1* may cause the altered regulation of this gene and the
346 vernalisation requirement in NOR1.

347 Origin of the vernalisation response in NOR1

348 NOR1 is the only woodland strawberry accession with a reported vernalisation requirement (Heide
349 & Sønsteby 2007). To obtain a more general view on the frequency of the vernalisation requirement
350 in Nordic countries, we subjected accessions from 67 locations, including NOR1 and FIN56 as
351 controls, to natural conditions in Helsinki during the early autumn of 2014 (Fig. S1), and then
352 moved plants into a glasshouse (LDs/20°C) for flowering observations. NOR1 was the only
353 accession that did not flower (Table S1), indicating that the vernalisation requirement observed in
354 NOR1 is rare in Nordic populations or even a unique response in a single population.

355 To test if NOR1 is endemic to northern Norway, we studied population structure of Nordic
356 accessions collected from 78 locations. PCA on GBS data showed that there were three main
357 genetic clusters: Iceland, Finland and the Alta region in Norway (Fig. 7). Eight samples from the
358 Alta fjord including NOR1, clustered with three samples from the adjacent fjord (Kvænangen). The
359 Finnish samples were closely related to each other, excluding the two most northern samples that
360 showed some similarity with the samples from the Alta region. The other Norwegian samples
361 comprised a genetically diverse group. For example, samples from Kåfjord and one sample from
362 southern Norway (Ås) were genetically the most similar to Finnish samples whereas samples from
363 Kvaløya near Tromsø clustered with the Icelandic samples. Also two samples from the central part
364 of Norway (Trøndelag) shared some genetic similarity with Icelandic samples. Our findings that
365 woodland strawberry populations present in the Alta region form a clear separate genetic cluster, in
366 which NOR1 is the only accession that requires vernalisation, strongly suggests that the
367 vernalisation requirement has evolved recently in the Alta region.

368 Discussion

369 As stated already by Heide & Sønsteby (2007), the arctic accession of woodland strawberry NOR1

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370 requires a prolonged period of cold before being able to receive flowering-promotive signals. Here,
371 we provide evidence based on gene expression analyses, crossing experiments, gene silencing and
372 genetic mapping that the vernalisation requirement is caused by the altered regulation of *FvTFL1*, a
373 gene that encodes a repressor of flowering that controls the yearly growth cycle in woodland
374 strawberry (Koskela *et al.*, 2012) as well as in several other species of the Rosaceae (Iwata *et al.*,
375 2012; Flachowsky *et al.*, 2012; Freiman *et al.*, 2012; Koskela *et al.*, 2016). We also report that
376 NOR1 has special environmental requirements during and following vernalisation in order to reach
377 the competence to flower and to initiate flowers, respectively. Based on genome-wide genotyping
378 and phenotypic data on a large set of North European woodland strawberry accessions, we suggest
379 that the mutation conferring the vernalisation requirement in NOR1 is unique and native to a single
380 location in northern Norway.

381 *FvTFL1* is needed for the vernalisation response in NOR1 and its silencing requires extreme
382 cold

383 In both diploid (FIN56) and octoploid species of *Fragaria*, SDs and/or cool temperatures around
384 10–13°C down-regulate *TFL1* (Nakano *et al.*, 2015; Rantanen *et al.*, 2015; Koskela *et al.*, 2016).
385 Regulation of *FvTFL1* in the arctic accession NOR1 contrasted with these earlier findings, as
386 *FvTFL1* expression was not suppressed as a response to SDs and 11°C (Fig. 1a). Even a
387 temperature of 4°C, that is well within the temperature range generally considered to fulfill the
388 vernalisation requirement (Duncan *et al.*, 2015), did not downregulate *FvTFL1* (Fig. S3a). In fact, a
389 prolonged period of near-freezing temperature was required to down-regulate this floral repressor in
390 the NOR1 accession (Figs 2 and S3b). The altered pattern of *FvTFL1* expression in NOR1
391 prompted us to study the effect of non-functional or silenced *FvTFL1* in F1 and F2 generations
392 resulting from crosses between wild type NOR1 and transgenic H4 and FIN56 carrying *FvTFL1*-
393 RNAi constructs.

394 In the two generations in both crosses, lack of *FvTFL1* expression was sufficient to abolish the
395 vernalisation requirement (Fig. S9 and Tables S5, S6). Further mapping in the NOR1 × FIN56 F2
396 generation showed that a 1-bp deletion at the 5’-promoter of *FvTFL1* co-segregates with the
397 vernalisation requirement (Fig. 5; Table S8), and it is the only polymorphism inside the mapping
398 window, which was found to fully correlate with vernalisation requirement in our population
399 samples. Moreover, expression analysis in plants from the NOR–P1 population confirmed that an

400 exposure to SDs at 11°C lowered the level of *FvTFL1* mRNA in plants without the deletion (Fig. 6).
401 Taken together, these data strongly indicate that the 1-bp deletion found in the promoter of *FvTFL1*
402 leads to altered regulation of the gene.

403 The deletion causes changes in several predicted transcription factor binding sites (Table 2). In
404 concordance with the altered photoperiodic response of *FvTFL1* in NOR1, this mutation abolishes a
405 DOF transcription factor binding site that is required for phytochrome-mediated light responses
406 (Weirauch *et al*., 2014) in vernalisation-requiring individuals. However, its role in the regulation of
407 *FvTFL1* requires functional validation.

408 If this mutation is the cause of the vernalisation requirement, it shares similarities with the
409 vernalisation mechanism described in beet. In annual beet, LDs up-regulate *BvBTC1*, which in turn
410 activates the floral promoter BvFT2, whereas biennial beet accessions are non-responsive to LDs
411 due to a mutation in the *BvBTC1* promoter (Pin *et al*., 2012). Biennial beet requires vernalisation
412 before *BvBTC1* expression returns to the level needed for allowing floral induction. Similarly,
413 *FvTFL1* in NOR1 may have lost the normal response to SDs and cool temperature due to the
414 mutation in the promoter region. As the mutated *BvBTC1* in beet, *FvTFL1* in NOR1 may require a
415 period of cold to be sufficiently downregulated to allow subsequent floral initiation. This hypothesis
416 is also compatible with the fact that the vernalisation-requiring phenotype of NOR1 is dominant
417 over the wild-type; just one *FvTFL1* allele derived from NOR1 is enough to promote *FvTFL1*
418 expression and inhibit flowering in non-vernalised plants.

419 Also in other species, long periods of cold are needed to silence different floral repressors,
420 including the MADS box transcription factor FLC/PEP1 in Brassicaceae and the CCT domain
421 protein in grasses, to make plants competent to respond to inductive signals (Michaels & Amasino,
422 1999; Yan *et al*., 2004; Wang *et al*., 2009). In Brassicaceae, the photoperiodic pathway genes *FT*
423 and *SOC1*, as well as *FT* ortholog *VRN3* in grasses, are repressed in non-vernalized plants
424 (Hepworth *et al*., 2002; Yan *et al*., 2006; Wang *et al*., 2011). In the woodland strawberry accession
425 NOR1 in contrast, the regulator causing vernalisation requirement does not affect the expression of
426 *FvFT1* and *FvSOC1*. These data indicate that the floral repressor in NOR1 functions downstream or
427 in parallel with *FvFT1* and *FvSOC1*. Indeed, previous studies showed that *FvTFL1* functions
428 downstream of FvFT1 and FvSOC1 (Koskela *et al*., 2012; Mouhu *et al*., 2013; Rantanen *et al*.,
429 2014), supporting the role of *FvTFL1* as a floral repressor causing the vernalisation requirement in

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430 NOR1. In *Arabis alpina*, AaTFL1 prevents flower initiation in young plants even after vernalisation
431 has silenced a major floral repressor, the FLC orthologue PEP1 (Wang *et al.*, 2011). However, FLC
432 orthologs are not found in woodland strawberry genome (Shulaev *et al.*, 2011), indicating that the
433 role of TFL1 homologs in the vernalisation responses of woodland strawberry and *Arabis alpina* are
434 different.

435 NOR1 requires cool post-vernalisation temperature to flower

436 The vernalisation response in NOR1 was first characterized by Heide & Sønsteby (2007), who
437 showed that NOR1 plants required at least 5 weeks of chilling at 2°C to weakly initiate
438 inflorescences. Similarly, the data presented here show that cold treatment alone is not sufficient for
439 floral initiation in NOR1, but vernalisation at near-freezing temperatures must be followed by cool
440 temperatures around 10°C to avoid de-vernalisation (Chouard, 1960), and/or to allow floral
441 initiation (Figs 3, 4, S8; Table 1). To our knowledge, this response has not previously been
442 characterised at the molecular level.

443 Although only exposure to 10°C after vernalisation at near-freezing temperatures lead to floral
444 initiation, similar levels of *FvTFL1* mRNA were detected in NOR1 apices after one to five weeks at
445 10°C and 20°C following vernalisation (Figs 4, S8). It remains unclear how NOR1 was able to
446 flower at 10°C despite *FvTFL1* being up-regulated. It is possible that a floral promoter is expressed
447 exclusively in cool conditions. A candidate for such a floral promoter could be *FvFT3*, whose up-
448 regulation was observed only in plants grown at 10°C, at the same time or slightly earlier than the
449 floral meristem identity gene *FvAP1* (Figs 3, S8). Likewise, Nakano *et al*. (2015) detected *FaFT3*
450 expression at an earlier time point than *FaAP1* in flower-inducing conditions in the octoploid
451 strawberry. Our finding that *FvFT3* is activated in SDs in both FIN56 and non-vernalisation
452 requiring NOR-P1 individuals, but not in NOR1 (Figs 1, 6) further supports its role in flower
453 induction. Taken together, our data is in line with the model that *FvFT3* is activated under flower-
454 inducing conditions, i.e. cool temperatures and/or SDs, after the silencing of *FvTFL1*. According to
455 this model, NOR1 would first require extreme cold during winter to suppress *FvTFL1* followed by
456 cool temperatures in the spring to activate *FvFT3*, whereas in FIN56, both silencing of *FvTFL1* and
457 the activation of *FvFT3* occur under the same conditions in autumn, leading to fundamental
458 differences in the developmental timing in these accessions (Fig. 8). More detailed temporal gene
459 expression analyses and the functional validation of *FvFT3* is needed to confirm its role.

460 **Vernalisation requirement may be beneficial in the northern climate**

461 The vernalisation response is an adaptive trait; in Arabidopsis, a cline in vernalisation sensitivity
462 has been observed, with northern accessions requiring a longer period of vernalisation than the
463 southern accessions (Stinchcombe *et al*., 2005). In beet, vernalisation response is restricted to the
464 cultivated forms and to northern accessions of *Beta vulgaris* ssp. *maritima*, the putative ancestor of
465 the cultivated beet (Pin *et al*., 2010). NOR1 that originates from the northern limit of the
466 geographical range of the *Fragaria* genus is the only known example within *Fragaria* that requires
467 vernalisation. We tested a total of 67 accessions collected from northern Europe, and found no other
468 genotypes that required vernalisation (Table S1). Moreover, genome-wide genotyping data on 78
469 accessions showed that NOR1 grouped together with the other accessions originating from the Alta
470 fjord in the north of Norway (Fig. 7). These data suggest that the mutation causing the NOR1
471 phenotype is a local one, and has probably arisen relatively recently after the last de-glaciation
472 event. The finding also supports the notion that the vernalisation requirement has arisen
473 independently in several individual plant lineages (e.g. Ream *et al*., 2012), and highlights the ability
474 of plants to adapt to different environments. In the extremely northern habitat of NOR1, a
475 mechanism for postponing the formation of flower buds until the spring may be an advantageous
476 trait. Similarly, this trait could be useful in the cultivated strawberry to avoid frost damage of flower
477 buds that commonly occurs during winter (Boyce *et al*., 1985).

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486 **Author contributions**

487 E. A. K., A. S., T. H. and O. M. H. designed and carried out the growth experiments. E. A. K.
488 genotyped the F2 populations, extracted RNA, performed RT-qPCR analysis, and carried out SEM.

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489 T. K. made the crosses between NOR1 and the other parents. T. H. and D. J. S. prepared GBS
490 libraries. T. T. carried out GBS and whole genome data analyses and population genomics. T. H.
491 phenotyped woodland strawberry accessions. S. I. participated in marker development. H. H. and L.
492 J. provided plant materials. T. H. and P. E. supervised the study. Manuscript was written by E. A. K.,
493 T. T. and T. H. with input from all the authors.

494 **Tables**

495 **Table 1.** Flowering time of woodland strawberry NOR1 plants grown at 10ºC or 22ºC following
496 vernalisation. The outdoor-vernalised NOR1 plants were moved indoors on January 5th. The
497 controlled climate plants were vernalised at ±2 ºC for ten weeks. In both experiments the vernalised
498 plants were then moved to 10ºC or 22ºC (LDs) for five weeks. Flowering was observed in
499 subsequent LDs (24 h, 20 ºC). Days to anthesis is expressed as days from the end of the
500 vernalisation period.
501

Vernalisation	Post-vernalisation temperature (ºC)	n	% flowering	Days to anthesis ± standard deviation**	No of inflor.	No. of flowers
Outdoors	10	12	75	54.6	1.4	12.9
	20	12	0	na	0	0
Controlled	10	10	100	55.2 ± 2.2	n/a	n/a
	22	6	0	na	0	0

502 **Mean of flowering plants only; na = not applicable; n/a = not analyzed
503

504 **Table 2**. Predicted promoter motifs in the 5'-promoter region of *FvTFL1* different between the
505 woodland strawberry accessions NOR1 and FIN56. Promoter motifs were predicted using
506 PlantPAN promoter database (Chow *et al*., 2015).

Hit sequence	Similarity score	Family	TF ID	TF function
FIN56 promoter motifs not found in the promoter region of NOR1				
ATCAA	0.8	AP2; ERF	AT3G14230	ethylene-responsive TF
ATCAA	0.8	motif sequence only		WRKY DNA binding site
TCAAG	0.8	motif sequence only		target site for trans-acting StDOC1 protein controlling guard-cell specific gene expression in potato
AAGGA	1	DOF		phytochrome-mediated light responses
NOR1 promoter motifs not found in the promoter region of FIN56				

tagATATCag	0.93	MYB; G2-like	AT2G20570	May function in photosynthetic capacity optimization
ATATCaggagtggattcg	0.72		X52153	unknown rice TF

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665 Figure legends

666 Figure 1. Expression of flowering related genes in LD and SD treated NOR1 and FIN56 accessions
667 of woodland strawberry. Relative expression of (a) *FvTFL1*, (b) *FvAP1,* (c) *FvFT1*, (d) *FvSOC1* and
668 (e) *FvFT3* in NOR1 and FIN56. *FvFT1* expression was studied in leaves and the expression of other
669 genes in shoot apices after 3-week treatments. Error bars present standard deviation between
670 biological replicates (n = 3). Asterisks indicate significantly different relative expression between
671 the two genotypes under the specified environmental conditions (Tukey's HSD, ** $p < 0.05$ and * p
672 < 0.1). LD = long day; SD = short day.

673 Figure 2. Effect of vernalisation on flowering related genes on NOR1 and FIN56 accessions of
674 woodland strawberry. Relative expression of (a) *FvTFL1* in shoot apices of NOR1 and FIN56
675 grown in LDs or in SDs at ±2°C. In (b–d), the plants were first grown in SDs for three weeks, after
676 which they were subjected to ±2°C, and the relative expression of (b) *FvTFL1*, (c) *FvSOC1* and (d)
677 *FvAP1* was studied in shoot apices. Error bars present standard deviation between biological
678 replicates (n = 3). Relative expression values denoted by the same letter do not differ significantly
679 by least-squares means at $p = 0.05$. SD = short day.

680 Figure 3. SAM morphology and gene expression in vernalized woodland strawberry accession
681 NOR1. (a) Morphology of NOR1 shoot apical meristems (sa – shoot apex, lp – leaf primordia, ls –
682 leaf stipule, fp – flower primordium, im2 – secondary inflorescence meristem, b – bract, s – sepal
683 primordia, p – petal primordia), and relative expression of (b) *FvAP1* and (c) *FvFT3* in shoot apex
684 samples. Plants were vernalized in the field until February 2017 and then grown in a growth
685 chamber in LDs at 10°C. Samples were collected immediately following vernalisation and after
686 one, two and five weeks. In (b) and (c), error bars denote standard deviation between biological
687 replicates (n = 3), and asterisks indicate statistically significant differences to week 0 (Tukey's
688 HSD, $p < 0.05$). In (a), the scale bars in W0, W1 and W2 shoot apices denote 150 µm, and in
689 W5, the scale bar is 300 µm. LD = long day.

690 Figure 4. Effect of post-vernalisation temperature on the expression of flowering related genes in
691 the woodland strawberry accession NOR1. Relative expression of (a) *FvTFL1*, (b) *FvSOC1* and (c)
692 *FvAP1* in apices of field-vernalised plants. Six-week-old plants were grown in the field at Kapp,

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693 Norway (60° 40’ N, 10° 52’ E) from September 2014 to January 2015 (Weather data Fig. S7) and
694 then subjected to 10°C or 20±2°C in 24-h LDs for five weeks in growth chambers. After
695 temperature treatments, plants were taken into a glasshouse (LDs 24 h/18–20°C) for flowering
696 observations. Error bars present standard deviation between biological replicates (n = 3).

697 Figure 5. Genetic mapping of the *VERN* locus in woodland strawberry. Genetic map (a) of the
698 NOR1×FIN56 F2 population and its correspondence to the Fvb reference genome. The map was
699 constructed based on the genotypes of 93 F2 individuals generated by SSR, Sequenom and HRM
700 markers. The markers indicated in green are polymorphisms identified between vernalisation-
701 requiring and non-requiring individuals from the Alta region in Northern Norway. (b) F_{ST} values of
702 all SNPs between four vernalisation-requiring and 13 non-requiring population samples within the
703 855 kb mapping window around the *VERN* locus. The SNP marked in red corresponds to the marker
704 SNP35. [color figure]

705 Figure 6. Gene expression in vernalisation-requiring and non-requiring individuals from the
706 woodland strawberry NOR-P1 population. Relative expression of (a) *FvTFL1*, (b) *FvAP1* and (c)
707 *FvFT3* in shoot apices of SD and LD grown individuals from the NOR-P1 population. NOR1
708 denotes vernalisation-requiring plants carrying the 1bp deletion and NOR P1-4 non-vernalisation
709 requiring plants without the deletion from the same population. Error bars denote standard deviation
710 between biological replicates (n = 3). Values marked by the same letter are not significantly
711 different by least-squares means (p = 0.05). LD = long day; SD = short day; n.d. = not detected;
712 N/A = not applicable.

713 Figure 7. Principal component analysis of genetic structure of Northern European woodland
714 strawberry. Geographic groups: yellow = Iceland, purple = Alta region, black = other Norway, red =
715 Southern Finland, green = Middle Finland, blue = Northern Finland. [color figure]

716 Figure 8. Schematic diagram of the contrasting seasonal cycles in NOR1 and FIN56 accessions of
717 woodland strawberry. The floral repressor *FvTFL1* (a) is down-regulated in FIN56 during autumn
718 as a response to short days (SDs) and cool temperatures below 13°C. In NOR1, the expression of
719 *FvTFL1* is slightly up-regulated in autumn and down-regulation occurs only after a sufficiently long
720 period of near-freezing temperature. In spring, cool temperature represses *FvTFL1* in FIN56 until
721 the temperature rises to approximately 15°C, after which LDs promote *FvTFL1* expression. The
722 floral meristem identity gene *FvAP1* (b) is up-regulated in FIN56 in autumn and its expression level

723 remains fairly stable throughout winter. In NOR1, up-regulation of *FvAP1* occurs only in over-
724 wintered plants in spring. LD = long day; SD = short day. [color figure]

725

726 ***New Phytologist* Supporting Information**

727 **Fig S1** Temperature in Helsinki, Finland in autumn 2014.

728 **Fig S2** Temperature in Helsinki, Finland in autumn 2016–winter 2017.

729 **Fig S3** Expression of flowering-related genes in NOR1 plants grown at 0°C or 4°C.

730 **Fig S4** Seasonal flowering in NOR1 and FIN56.

731 **Fig S5** Weather conditions in Helsinki, Finland 2013–2014.

732 **Fig S6** Temperature at Kapp, Norway in autumn/winter 2014-2015.

733 **Fig S7** Flower buds in NOR-P1 individuals immediately after vernalisation.

734 **Fig S8** Gene expression in artificially vernalised NOR1 plants.

735 **Fig S9** Flowering in NOR1 × H4 F1 plants.

736 **Fig S10** Linkage map of eight SSR markers genotyped in 534 F2 NOR1 x FIN56 plants.

737 **Fig S11** Linkage map of 27 loci genotyped in 354 F2 individuals from the NOR1 x FIN56 cross.

738 **Fig S12** Sequences of the fragment amplified by SNP35.

739 **Fig S13** *FvTFL1* sequence in NOR1 and FIN56.

740 **Table S1** *F. vesca* accessions used for estimating population structure and phenotype**.**

741 **Table S2** Primers used in the study.

742 **Table S3** Sequenom primers used in the study.

743 **Table S4** Flowering of NOR1 and FIN56 plants exposed to SDs or LDs.

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744 **Table S5** Flowering phenotypes in NOR1 × FIN56 F2 population.

745 **Table S6** Flowering in non-transgenic seedlings of NOR1 × H4 F2 population.

746 **Table S7** Polymorphisms within the NOR1 population.

747 **Table S8** Markers flanking the *VERN* locus.

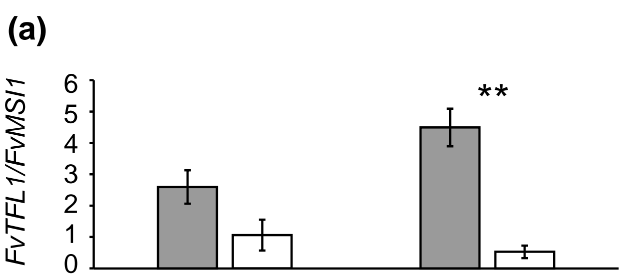
748 **Methods S1** Protocol for SSR analysis.

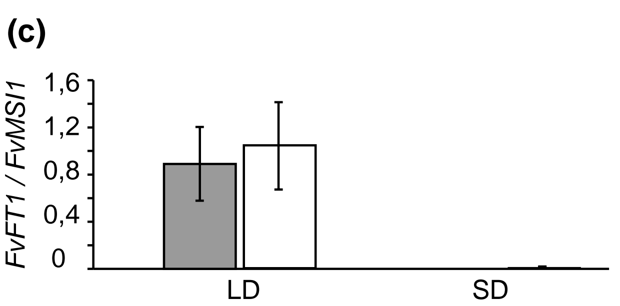
749 **Methods S2** Protocol for Sequenom genotyping.

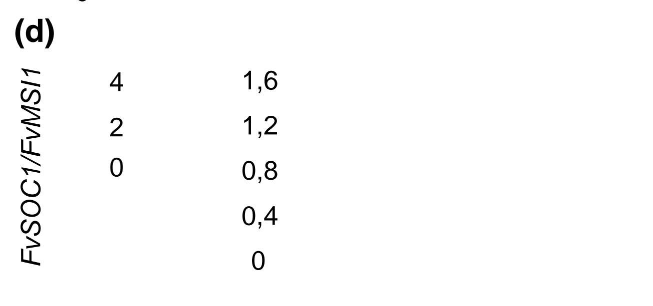
750 **Methods S3** Protocol for HRM genotyping.

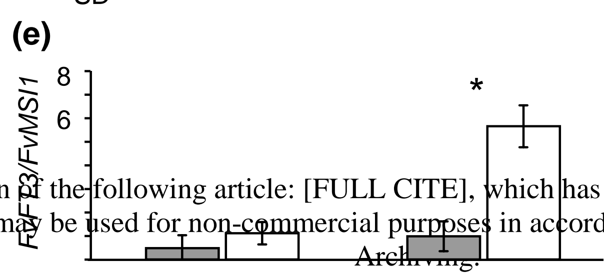
751 **Methods S4** Illumina whole genome sequencing.

752 **Notes S1** The NOR1 × H4 cross.









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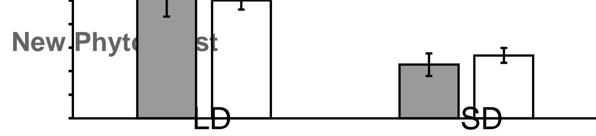
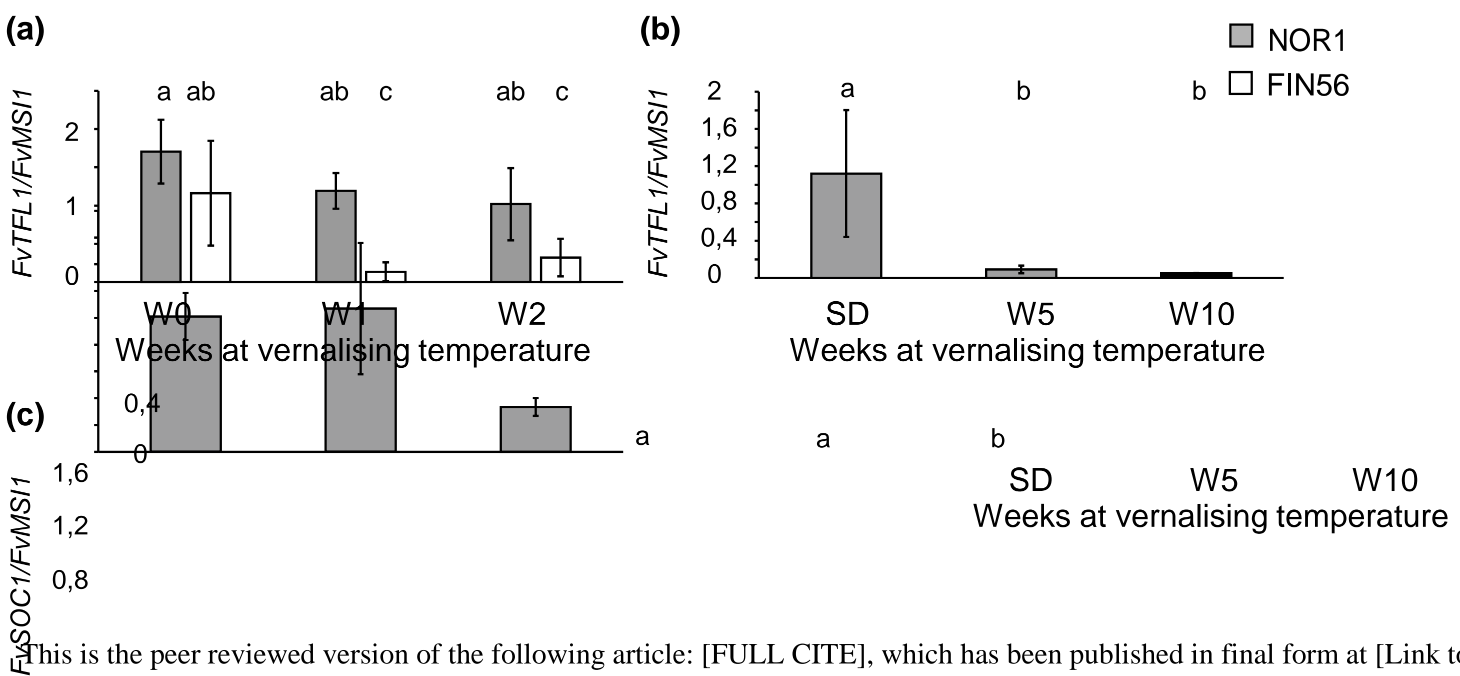


LD SD



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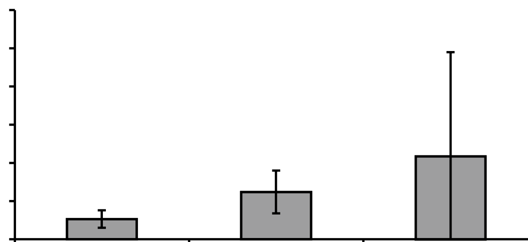
FvAP1/FvMSI1



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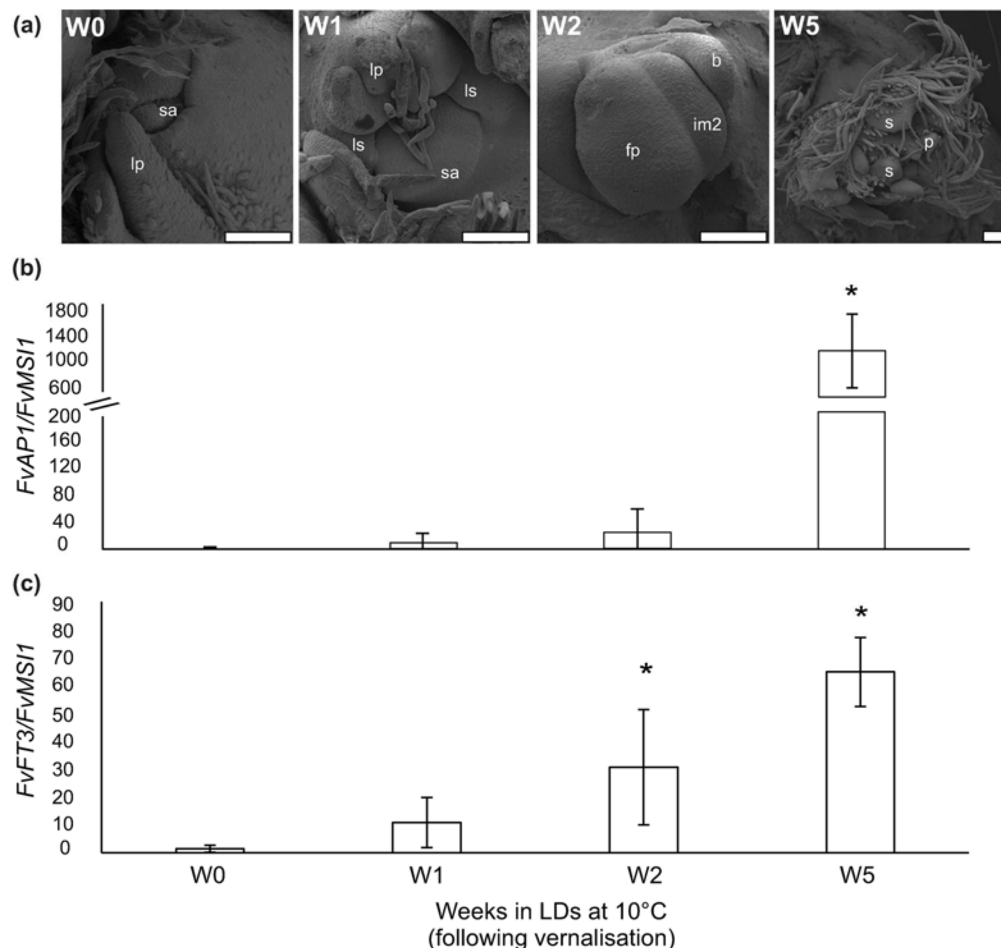

Figure 3. SAM morphology and gene expression in vernalized NOR1 plants. (a) Morphology of NOR1 shoot apical meristems (sa – shoot apex, lp – leaf primordia, ls – leaf stipule, fp – flower primordium, im2 – secondary inflorescence meristem, b – bract, s – sepal primordia, p – petal primordia.), and relative expression of (b) FvAP1 and (c) FvFT3 in shoot apex samples. Plants were vernalized in the field until February 2017 and then grown in a growth chamber in LDs at 10°C. Samples were collected immediately following vernalisation and after one, two and five weeks. In (b) and (c), error bars denote standard deviation between biological replicates ($n = 3$), and asterisks indicate statistically significant differences to week 0 (Tukey's HSD, $p < 0.05$).

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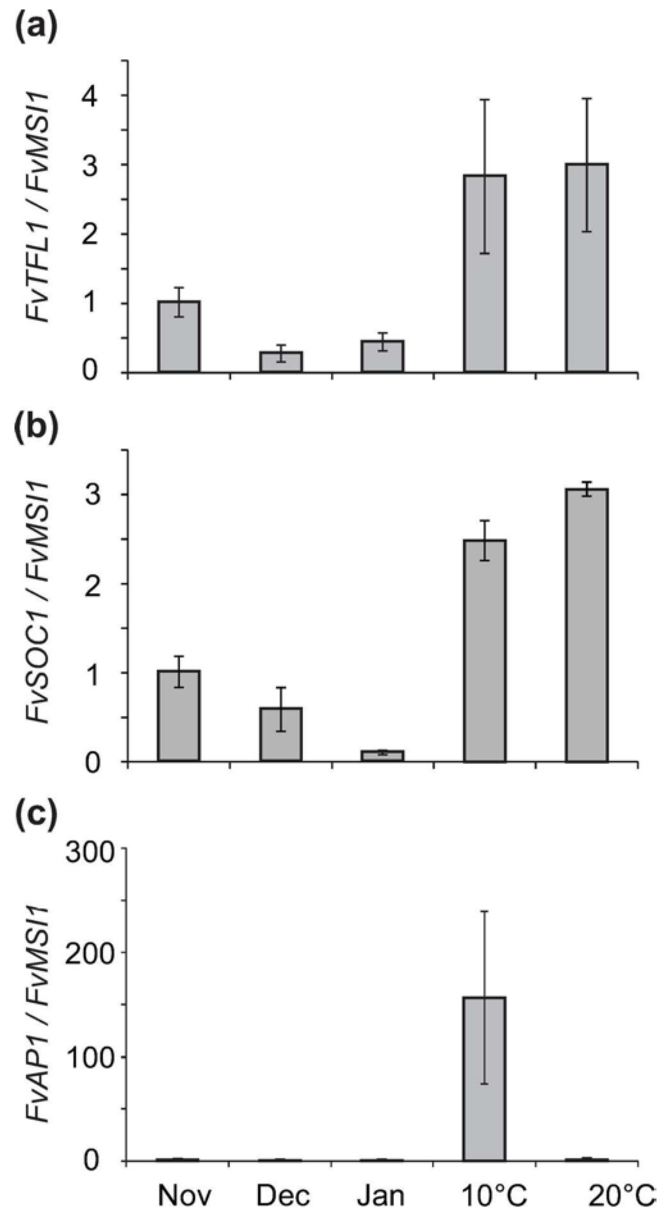


Figure 4. Effect of post-vernalisation temperature on the expression of flowering related genes in NOR1. Relative expression of (a) FvTFL1, (b) FvSOC1 and (c) FvAP1 in apices of field-vernalised plants. Six-week-old plants were grown in the field at Kapp, Norway (60° 40′ N, 10° 52′ E) from September 2014 to January 2015 (Weather data Fig. S7) and then subjected to 10°C or 20±2°C in 24-h LDs for five weeks in growth chambers. After temperature treatments, plants were taken into a glasshouse (LDs 24 h/18–20°C) for flowering observations. Error bars present standard deviation between biological replicates (n = 3).

138x259mm (300 x 300 DPI)

(a)

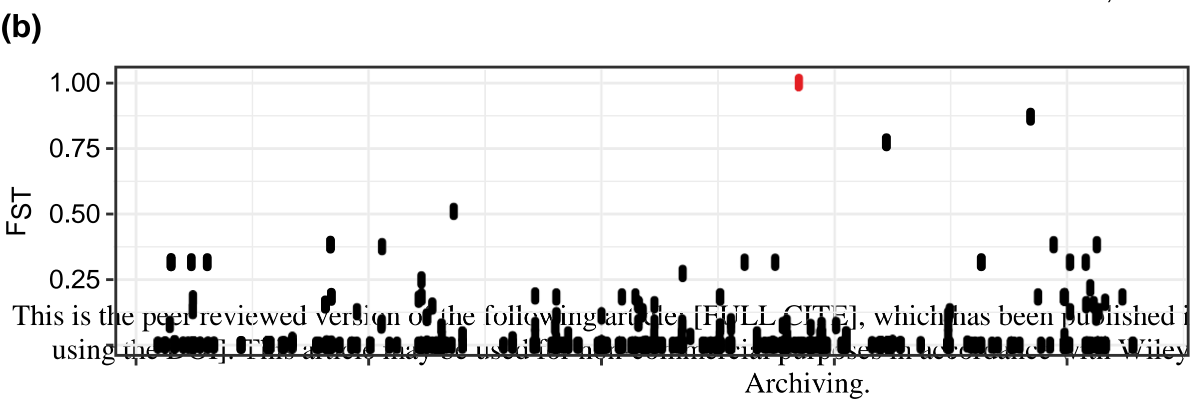
LG6
linkage map

LG6
scaffolds

cM	Marker
0.0	SNP27
7.3	FVB6_1986765 SNP28
10.2	FVB6_2497098
19.8	SNP29 SNP31
	SNP32
23.3	FVB6_4471366
31.6	EMFn185
40.7	FAES0082 SCAR2
	SNP34 FVES0035
41.8	**VERN**
41.9	SNP35
43.5	FVB6_13701141 FVB6_17781611
45.2	FVB6_17826511
45.8	FVES0015
46.3	FVB6_18668797
46.9	FVB6_18364274 FVB6_19430764
	FVB6_13481846 SNP42
61.8	SNP41
78.0	SNP43
83.1	SNP36 Fvi020
	SNP38
88.3	FVB6_24280305
91.3	FVB6_25456914
99.5	SNP44 FVB6_30289324
101.4	FVES2142
104.0	FVB6_15246561
111.7	FVB6_32296038 FVB6_31783993
113.4	SNP45
119.0	FVB6_33318755
134.0	FVB6_36298576
144.4	UFFxA01E03-HEX
153.2	SNP47

38,87 Mb

(b)



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0.00
9.8 10.0 10.2 10.4 10.6
Mb

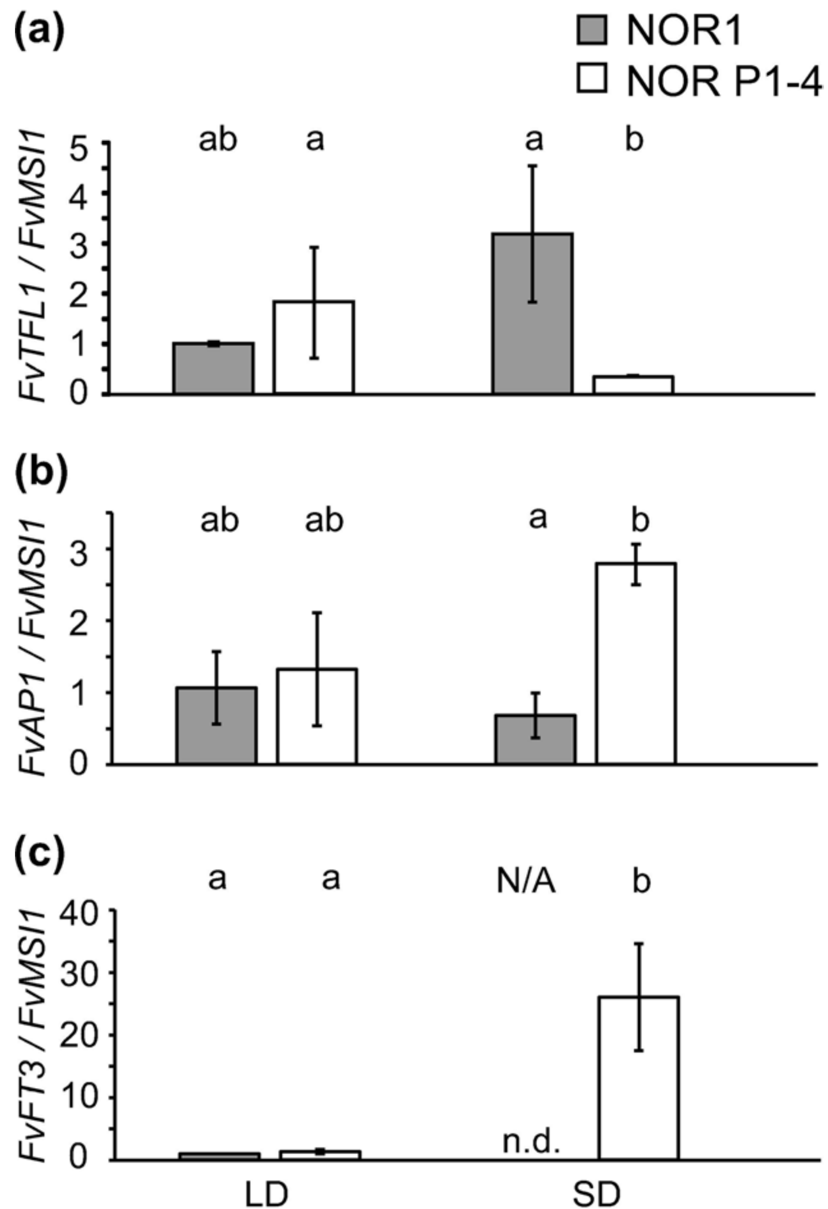

Figure 6. Gene expression in vernalisation-requiring and non-requiring individuals from the NOR-P1 population. Relative expression of (a) FvTFL1, (b) FvAP1 and (c) FvFT3 in shoot apices of SD and LD grown individuals from the NOR-P1 population. NOR1 denotes vernalisation-requiring plants carrying the 1bp deletion and NOR P1-4 non-vernalisation requiring plants without the deletion from the same population. Error bars denote standard deviation between biological replicates (n = 3). Values marked by the same letter are not significantly different by least-squares means (p = 0.05)

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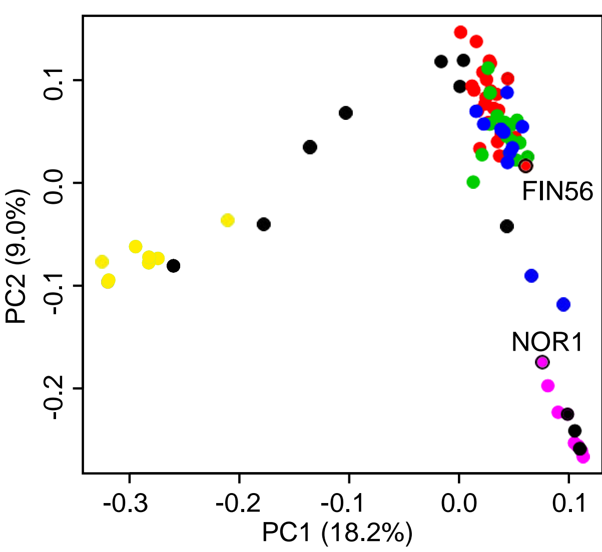
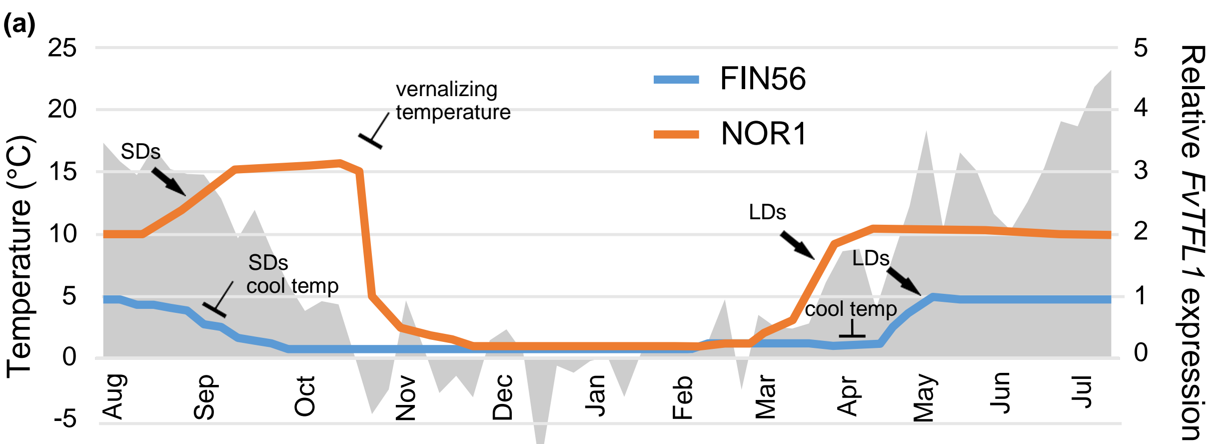
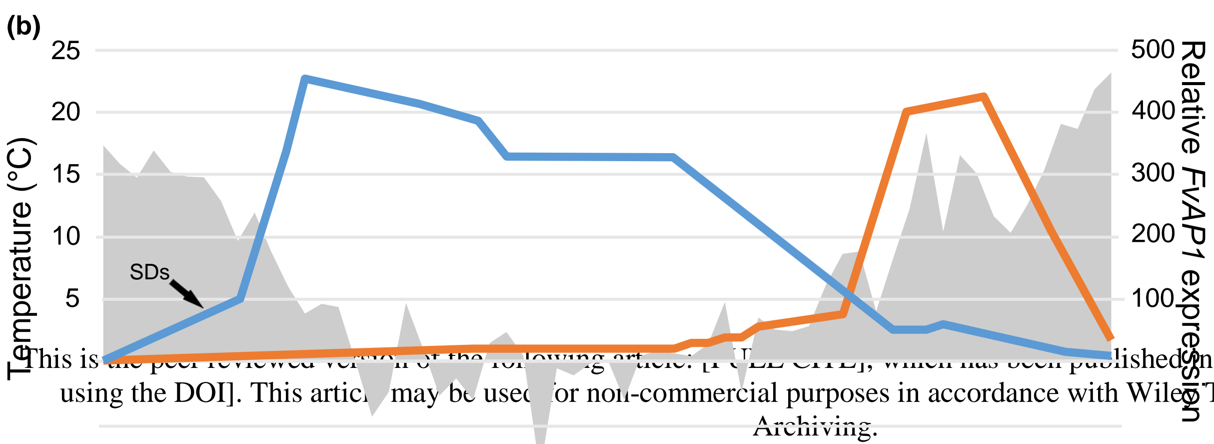

FIN56
NOR1
PC2 (9.0%)
PC1 (18.2%)
0.1
0.0
-0.1
-0.2
-0.3
-0.2
-0.1
0.0
0.1





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