Functional and expressional analyses of apple *FLC-like* in relation to dormancy progress and flower bud development

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Running head: Growth inhibiting function of apple *FLC-like*

Abstract

We previously identified the *FLOWERING LOCUS C* (*FLC*)-*like* gene, a MADS-box transcription factor gene that belongs to *Arabidopsis thaliana FLC* clade, in apple (*Malu*s x *domestica*), and its expression in dormant flower buds is positively correlated with cumulative cold exposure. To elucidate the role of the *MdFLC-like* in the dormancy process and flower development, we first characterized the phenotypes of *MdFLC-like* overexpressing lines with the *Arabidopsis* Columbia-0 background. The overexpression of *MdFLC-like* significantly delayed the bolting date and reduced the plant size, but it did not significantly affect the number of rosette leaves or flower organ formation. Thus, *MdFLC-like* may affect vegetative growth and development rather than flowering when expressed in *Arabidopsis*, which is not like *Arabidopsis FLC* that affects development of flowering. We compared seasonal expression patterns of *MdFLC-like* in low-chill 'Anna' and high-chill 'Fuji' and 'Tsugaru' apples collected from trees grown in a cold winter region in temperate zone, and found an earlier up-regulation in 'Anna' compared with 'Fuji' and 'Tsugaru'. Expression patterns were also compared in relation to developmental changes in the flower primordia during the chilling accumulation period. Overall, *MdFLC-like* was progressively up-regulated during flower primordia differentiation and development in autumn to early winter, and reached a maximum expression level at around the same time as the genotype-dependent chilling requirements were fulfilled in high-chill cultivars. Thus, we hypothesize *MdFLC-like* may be up-regulated in response to cold exposure and flower primordia development during the progress of endodormancy. Our study also suggests *MdFLC-like* may have a growth inhibiting function during the end of endodormancy and ecodormancy, when the temperature

is low and unfavorable for rapid bud outgrowth.

Keywords; bud dormancy, chilling requirement, FLC, flower development, MADS-box transcription factor, *Malus* × *domestica*

Introduction

Perennial woody plant species native to temperate zones modulate their growth to correspond with seasonal environmental changes and often suspend growth during the winter. This process is known as dormancy and is considered an adaptive process that enables plants to survive environmental stresses, such as low temperature. Lang (1987) and Lang et al. (1987) defined plant dormancy as “the temporary suspension of visible growth of any plant structure containing a meristem” and classified the fruit tree bud dormancy states as paradormancy, endodormancy, and ecodormancy. Both paradormancy and endodormancy are states induced by the perception of environmental or endogenous signaling cues, but they differ in whether they originate solely from meristem-containing tissue (endodormant) or from structures distinct from that undergoing dormancy (paradormant). A certain specific amount of chilling exposure is critical for inducing the shift from endodormancy to ecodormancy, known as the “chilling requirement for endodormancy completion and release”. Ecodormancy is a state brought about by a limitation in growth-promoting factors, such as warm conditions and the availability of water and nutrients. Whether buds are endodormant or ecodormant has been determined according to the competency of bud break, which is often based on the increase and decrease in the mean-time until bud break or in bud break percentage under forcing conditions. For example, genotype-dependent chilling requirements for endodormancy release are often considered satisfied at a specific sampling time point, if the bud break frequency increased over a threshold (often 50%) under forcing conditions (Bielenberg et al. 2015, Kitamura et al. 2018). Although Lang’s definition has been widely adopted by horticultural researchers, recently

accumulated data suggest that this terminology may need to be revised (Cooke et al. 2012, Considine and Considine 2016, Yamane, 2014). Apple (*Malus* × *domestica*), an economically important fruit crops, has adapted to the cool climate of temperate regions. In typical apple cultivars, flower meristem initiation occurs in early summer after terminal bud set (Kotoda et al., 2010). Then, terminal flower buds enter a dormant state in winter until blooming in spring. Typical characteristics of apple dormancy are that dormancy is induced and released in response to low temperature independent of photoperiod (Heide and Prestrud 2005). To date, the molecular mechanism underlying apple dormancy has been studied with a focus on specific gene(s) and gene network expression level changes (Falavigna et al. 2019, Kumar et al. 2016, Porto et al. 2015, Saito et al. 2017, Wang et al. 1991, Wisniewski et al. 2015) as well as the genetic control of bud break and blooming date (Allard et al. 2016, van Dyk et al. 2010, Celton et al. 2011, Miotto et al. 2019, Trainin et al. 2016, Urrestarazu et al. 2017).

We previously conducted RNA-sequencing (RNA-Seq) studies and identified a limited number of genes that were strongly associated with chill-unit accumulation under natural and cold-treated conditions using a strict candidate-gene selection strategy (Takeuchi et al. 2018). A highly significantly correlated gene was a MADS-box gene (hereafter *MdFLC-like*; *MD09G1009100*) found in a clade that included *FLOWERING LOCUS C* (*FLC*) and *MADS AFFECTING FLOWERING* (*MAF*) genes. *Arabidopsis FLC* represses the transcription of floral integrator genes, such as *FT* and *SOC1*, thus acting as a floral repressor to delay the development of floral buds and bolting, resulting in the increased number of leaves in flowering and bolting (Searle et al. 2006). In *Arabidopsis*, all the

84 genes in the *FLC* clade have the ability to repress the flowering pathway (Ratcliffe et al. 2001, Gu et
85 al. 2013). Also, an *FLC* ortholog of *Arabis alpine*, *PEP1*, functions in the return to vegetative
86 development after flowering, implying the significance of the *FLC* orthologs in the plant perennial life
87 cycle (Wang et al. 2009). Moreover, the involvement of *MAF3-like* homologs in dehydration-induced
88 endodormancy regulation was reported in leafy spurge (Dogramaci et al. 2014).

89 The expression of *MdFLC-like* is positively correlated with prolonged chilling exposure in
90 apple flower buds (Porto et al. 2015, Takeuchi et al. 2018). In contrast, although *Arabidopsis FLC* is
91 up-regulated in response to endogenous factors and environmental cues such as low temperatures
92 (Aikawa et al. 2010; Gu et al. 2013), it is down-regulated by prolonged cold during the vernalization
93 process (Michaels and Amasino, 1999). The up-regulation of the *FLC*-like gene's expression
94 concomitant to cumulative low temperature exposure has been found not only in apple, but also in
95 other Rosaceae perennial species such as *Prunus pseudocerasus* (Zhu et al. 2015) and *Taihangia*
96 *rupestris* (Du et al. 2008). Consequently, *FLC-like* genes may have unique functions in the perennial
97 life cycles in Rosaceae; however, the biological function of *MdFLC-like* has yet to be investigated.
98 Here, we conducted a functional characterization of *MdFLC-like* using *Arabidopsis* overexpression
99 lines to determine whether *MdFLC-like* is functionally similar to *Arabidopsis FLC*. We also compared
100 gene expression patterns across three apple cultivars, 'Anna', 'Fuji', and 'Tsugaru', which have
101 different dormancy behaviors, to analyze the expression changes of *MdFLC-like* in relation to chilling
102 requirement fulfillment, dormancy progress during endodormancy, the transition from endodormancy
103 to ecodormancy, and the temperature changes during ecodormancy. Furthermore, expression patterns

were also compared in relation to developmental changes in the flower primordia during the chilling accumulation period.

Materials and Methods

Transformation

MdFLC-like-specific primers were designed based on the published sequences (*MD09G1009100*; Daccord et al. 2017) as follows: mdFLC1-pGWB2-for (5'-[CACGGGGACTCTAG]AATGGGGCGAGGGAAGGTG-3') and mdFLC1-pGWB2-rev (5'-[GATCGGGGAAATTCGAGCT]CTTCAAAACAATTGTAGTATGGTGGC-3'). The full-length coding sequence of *MdFLC1* was amplified from 'Fuji' dormant buds cDNAs using the PrimeStar GXL (TaKaRa) and the primers listed above. Amplified fragments were cloned into the *Xba*I- and *Sac*I-digested pGWB2 vector (Nakagawa et al. 2007), placing the gene under the *Cauliflower mosaic virus* 35S promoter, using an In-Fusion HD cloning kit (TaKaRa). The sequences between brackets in the primers listed above were used for the recombination. The resultant 35S:*MdFLC-like* plasmid was verified by Sanger-sequencing and then transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Agrobacterium-mediated transformation of *Arabidopsis* Columbia-0 was performed using the floral dip method, as described previously (Zhang et al. 2006). Seeds of the transformed plants were sterilized with 70% ethanol, washed in purified water, and then placed on 0.1% agar containing 50 μg/mL kanamycin. After incubation in the dark at 4°C for 4 days, seeds were selected under long

124 photoperiod conditions (16-h light/8-h dark photoperiod) at 22 °C. We also confirmed the
125 transformation by the PCR amplification of *MdFLC-like*. The selected plants were transplanted into a
126 standard soil mixture soon after cotyledon expansion, and grown in a wrapped pot to maintain a high-
127 humidity level for the initial 3 days. Plants were then grown under a long photoperiod conditions.
128 Bolting date, flowering date, and the number of rosette leaves at bolting and flowering were recorded
129 for each transformed plant.
130
131 *Plant materials*
132 Apple plant materials used in this study were collected from trees planted on Apple Research
133 Station, Institute of Fruit Tree and Tea Science, NARO, Morioka, Japan (39°8’N, 141°1’E, 193-m
134 altitude). Morioka is located in the northern part of Japan. This area is in temperate zone with cold
135 winter but not extreme cold subarctic and boreal zone, which is suitable region for apple production.
136 The annual mean air temperature is 10.2°C. In the first winter season in this study (2016–2017), we
137 collected shoots less than approx. 40 cm bearing flower buds at terminal positions from the high-chill
138 Japanese cultivar ‘Fuji’ and low-chill Israeli cultivar ‘Anna’ throughout the dormancy process. In the
139 second season (2017-2018), we collected shoots in the same manner from ‘Anna’ and high-chill
140 Japanese cultivar ‘Tsugaru’. The chill unit (CU) on the sampling date was calculated according to the
141 Utah model (Richardson et al. 1974), which is calculated based on the hourly highest temperatures
142 recorded in the field of Tohoku Agricultural Research Center, Morioka, Japan (39°8’N, 141°1’E, 176
143 m altitude). This center is located at approximately 1520 m distance from sample collection site. On

144 each sampling date, flower buds dissected from collected shoots were immediately frozen in liquid
145 nitrogen and stored at −80°C until used. To analyze the expression changes of *MdFLC-like* in response
146 to warm temperatures, ~40-cm shoots of 'Fuji' and 'Anna' collected at the 1200 CU sampling point
147 were incubated under forcing conditions (22°C and a 16-h light/8-h dark photoperiod). The terminal
148 flower buds were then sampled just before bud burst occurred and frozen in liquid nitrogen and stored
149 at −80°C until used.
150
151 *Dormancy status evaluation*
152 At least five shoots were collected on each sampling date, artificially defoliated when leaves
153 were attached, and incubated in a growth chamber under forcing conditions (22 °C and a 16-h light/8-
154 h dark photoperiod). The basal parts of shoots were then soaked in water containing 1% (v/v) cut
155 flower preservative reagent (Misakifarm; Otsuka Kagaku, Tokushima, Japan). The water was changed
156 and the basal ends of the shoots were cut every week. The terminal flower bud burst was recorded
157 every week for four weeks under forcing conditions. The bud burst was defined as occurring when
158 green leaf tips became visible and were the same length as the derived dormant bud. The timing of the
159 end of endodormancy (chilling requirement fulfilled) was defined when over 50% of the terminal bud
160 burst had been observed under forcing conditions within 4 weeks.
161
162 *RNA extraction and qPCR*
163 Total RNA from apple flower buds was extracted using the PureLink Plant RNA Reagent

(Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Then, 600 ng of total RNA was reverse-transcribed using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo). Quantitative RT-PCR (qPCR) was conducted using LightCycler 480 (Roche) and THUNDERBIRD® SYBR qPCR Mix (Toyobo). The expression level of *MdFLC-like* was analyzed using primers FLC_qPCR_for (5'-GGAGGAGCGGGCTTATCAAG-3') and FLC_qPCR_rev (5'-TTGGCGGAGAAGATGACGAG-3'). qPCR for *MdFLC-like* was performed under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. Gene-specific amplification was confirmed using a melting curve. *SAND* was used as the reference gene as described previously (Imai et al. 2014). Three to five independent samples (flower buds) were used as biological replicates with one technical replicate each for each measurement.

Comparative observation of flower primordia differentiation and development

In the first season, flower bud samples collected at 0 CU (Sept. 21st, 2017), 400 CU (Nov. 9th, 2017) and 800 CU (Dec. 25th, 2017) from 'Fuji' and 'Anna' were fixed in FAA (1:1:18 chroloform:acetic acid:70% ethanol) and stored at 4°C until used. Then, flower bud samples (n=5) were incubated sequentially in 10%, 20%, and 30% sucrose for 2 h to overnight, then frozen in SCEM embedding solution (Leica). Frozen samples were sectioned every 10 μm, following the protocol of Kawamoto's film method (Kawamoto 2003) using a cryostat (Leica, CM1520). The sections were stained with 0.5% toluidine blue for 5 min, embedded in SCMM solution (Leica) and observed using a light microscope (Olympus, BX60).

Results

Functional characterization of MdFLC-like *in transgenic Arabidopsis*

To assess the functional conservation between *MdFLC-like* and *Arabidopsis FLC* in flowering, the coding sequence driven by the Cauliflower mosaic virus 35S promoter was introduced into *Arabidopsis*. In total, 10 independent transgenic lines were obtained and monitored for their growth and flowering behaviors. Significant delays in bolting and subsequent flowering were observed in transgenic lines (Figure 1A, B), while their rosette leaf numbers did not differ (Figure 1C, D). *MdFLC-like* did not appear to be involved in blooming retention in *Arabidopsis*, because the differences in the number of days to bolting and flowering were not significantly different with each other (Figure 1A, B). Flower organ morphology and differentiation were not affected by *MdFLC-like* overexpression (data not shown). Phenotypic changes observed in the transgenic *Arabidopsis* included smaller overall plant size (Figure 2A, B) and increased number of scapes compared with those of WT (Figure 2C-E).

Dormancy status of 'Anna', 'Fuji', and 'Tsugaru'

Under our experimental conditions, the terminal flower buds of 'Anna' burst in forcing conditions during all sampling periods, suggesting that this cultivar has very low potential to enter the endodormancy state (Figure 3). However, the bud burst rate in 'Anna' was increased by chilling accumulation, and reached almost 100% at 600 CU or above. For 'Fuji' and 'Tsugaru', no bud burst

was observed in the shoots at 600 CU or less, except ‘Tsugaru’ which showed a 25% bud burst at 0 CU. Bud burst rate was greater than 50% at 986 and 754 CU for ‘Fuji’ and ‘Tsugaru’, respectively (Figure 3). The days required for bud burst under forcing conditions decreased with CU accumulation in all three cultivars analyzed (Figure 3).

Expression changes of MdFLC-like *during chilling requirement fulfillment and in response to warm temperatures*

To study the potential involvement of *MdFLC-like* in the dormancy process, its expression changes throughout the process in apple cultivars with different chilling requirements were analyzed over 2 years. The expression of *MdFLC-like* initially increased with CU accumulation at the beginning of winter, and then remained at its maximum level or gradually decreased in all three cultivars in both seasons (Figure 4). The expression in the low-chill ‘Anna’ reached a maximum earlier than in the high-chill ‘Fuji’ in the 2016–2017 season, and a similar tendency was observed in comparison with high-chill ‘Tsugaru’ in the 2017–2018 season. The time in which the *MdFLC-like* expression reached its maximum appeared to match that in which the cultivar-dependent chilling requirement was fulfilled especially in ‘Fuji’ and ‘Tsugaru’.

To assess the warm temperature sensitivity of *MdFLC-like* in the dormant buds, its expression in terminal flower buds under forcing conditions was evaluated. After shoots that were collected at 1,200 CU were exposed to warm temperatures, we observed a significant decrease in the expression of *MdFLC-like* in terminal flower buds (Figure 5). This decreased expression trend was

observed both in ‘Anna’ and ‘Fuji’, regardless of the difference in the amounts of chilling requirement.

Comparative morphological observation of flower primordia at 0, 400, and 800 CU between ‘Fuji’ and ‘Anna’

Among the observed flower buds at 0 and 400 CU, those of ‘Anna’ had larger central and lateral flowers compared with ‘Fuji’ (Figure 6), suggesting that flower meristem differentiation and development began earlier and/or proceeded more rapidly in ‘Anna’ than in ‘Fuji’ during autumn at the sampling site. At 800 CU, the developmental stages of the central and lateral flowers appeared to be similar between ‘Anna’ and ‘Fuji’ (Figure 6B). A temporal suspension of flower development was observed after flower primoridia differentiation was completed in ‘Anna’ from 400 CU to 800 CU, whereas inflorescent meristem development appeared to progress continuously from 0 to 800 CU in ‘Fuji’ under our experimental conditions.

Discussion

MdFLC-like inhibited vegetative growth in Arabidopsis

The overexpression of *FLC* in *Arabidopsis* results in an extreme delay in flowering and an increase in the number of rosette leaves at bolting (Michaels and Amasino 1999). Similary, *Arabidopsis* lines overexpressing *MdFLC-like* also required more time to set flowers. The numbers of rosette leaves, however, were not increased at flowering and the overall plant sizes were significantly

244 smaller than the controls. These results implied that *MdFLC-like* is involved in the inhibition of
245 vegetative growth rather than flowering as like *Arabidopsis FLC*. Deng et al. (2011) raised the
246 possibility that *Arabidopsis FLC* may regulate not only flowering but also other developmental
247 pathways by changing binding partners. Indeed, our amino acid sequence alignment indicated that
248 some amino acid sequence set in the k-box region is deleted whereas those in the MADS-box domain
249 are highly conserved in MdFLC-like compared to other FLC homologs of apple (Supplementary Fig.
250 1). This suggests that *MdFLC-like* protein may have an ability to form different protein-protein
251 complexes from those of other FLC homologs. Further studies will be needed to clarify the mode-of-
252 action of the MdFLC-like functionality in apple. The study additionally showed that the
253 overexpression of *MdFLC-like* resulted in the rapid induction of secondary scapes, which may suggest
254 that *MdFLC-like* modulates the plant architecture possibly by affecting phytohormone levels.
255 Consequently, we hypothesize that *MdFLC-like* affects vegetative growth rather than reproductive
256 development in *Arabidopsis* and may act as a general growth regulator.
257
258 *MdFLC-like was highly expressed when genotype-dependent chilling requirement was fulfilled*
259 We first confirmed the very small amount of chilling requirement in low-chill 'Anna' based
260 on bud burst behavior under forcing conditions during seasonal chilling accumulation. 'Anna' is an
261 Israeli low-chill cultivar that needs 200–300 h below 7.2 °C to break bud dormancy, which is estimated
262 by the accumulated chilling that occurs until the initial bud burst under field conditions (Brooks and
263 Olmo 1972). Here, 'Anna' showed a lower chilling requirement compared with normal commercial

264 cultivars in Japan, although exact amount of chilling requirements for ‘Anna’ grown in Morioka, Japan,
265 appeared to be even lower than 200 CU. The chilling requirements of ‘Fuji’ and ‘Tsugaru’ were
266 estimated to be approximately 800−1,000 CU, which is consistent with previous studies reporting that
267 the chilling requirement for a greater than 50% bud break under forcing conditions for ‘Fuji’ grown in
268 Nagano Prefecture, Japan was ~800 CU (Takeuchi et al. 2018).

269 Seasonal expression patterns of *MdFLC-like* were positively correlated with low
270 temperature accumulations in apple cultivars having different chilling requirements in our 2-year’s
271 experiment, which is consistent with our previous findings (Takeuchi et al., 2018). Even under the
272 fluctuating climatic conditions, *MdFLC-like’*s expression appeared to peak near the time when the
273 cultivar-dependent chilling requirement was fulfilled especially in high-chill cultivars, suggesting the
274 robust control of expression during endodormancy until chilling requirement fulfillment, which may
275 occur through the sensing of chilling accumulation. In addition, *MdFLC-like* was down-regulated by
276 warm temperature (Figure 5). In *Arabidopsis halleri*, seasonal changes in *FLC* expression are
277 temperature-dependent and robustly controlled by past temperatures (Aikawa et al. 2010). Thus, apple
278 *MdFLC-like* and *Arabidpsis FLC* may be controlled by a conserved temperature-sensing regulatory
279 system.

280

281 *Differences in flower primordia differentiation may relate to the differences in* MdFLC-like
282 *expressions in low and high-chill cultivars before chilling requirement fulfillment?*

283 In apple, the floral meristems are believed to continue to develop during the period when

284 the terminal bud break is repressed (Kurokura et al., 2013). Thus, we hypothesize that differential
285 expression of *MdFLC-like* between low-chill and high-chill cultivars before chilling requirement
286 fulfillment may reflect differences in not only temperature response but also internal structures of
287 flower buds. To test this hypothesis, we compared the morphological changes in flower primordia
288 between low-chill 'Anna' and high-chill 'Fuji' at 0–800 CU. Interestingly, the obvious suspension of
289 flower primordia development has been observed in 'Anna' but not in 'Fuji' during chilling
290 accumulation until the late stage of endodormancy. Central and lateral flower sizes of 'Fuji' continue
291 to increase from 0 to 800 CU when the flower size became comparable to that of 'Anna'.
292 During 0–800CU, *MdFLC-like* was up-regulated earlier and faster in 'Anna' compared to
293 'Fuji' (Figure 4A). This appeared to be comparable to earlier and faster flower primordia development
294 in 'Anna' compared to 'Fuji' (Figure 6). Our study also revealed that *MdFLC-like* did not affect
295 *Arabidopsis* flowering and rather inhibited vegetative growth (Figure 1), which does not support the
296 idea that *MdFLC-like* control flower development in apple. Alternatively, our study collectively may
297 suggest that *MdFLC-like* expression is controlled in response to flower primordia development.
298 Further comparative expression studies using vegetative tissues and juvenile tissues may help clarify
299 the involvement of flower development in regulatory mechanism of *MdFLC-like* expression.
300
301 *Apple flower development properties in comparison with* Prunus *fruit trees*
302 As 'Fuji' apple shown in this study, peach (*Prunus persica*) in the Rosaceae, the same family
303 as apple belongs to, flower meristem continues to develop during late autumn and winter dormancy

304 (Reinoso et al. 2002). However, the progress of flower primordia development in low-chill cultivars
305 in comparison with high-chill cultivars appeared to be different between apple (this study) and *Prunus*
306 fruit trees. Our microscopic observations suggested that flower differentiation and development is
307 rather advanced in low-chill 'Anna' compared to high-chill 'Fuji' at 0 and 400 CU. Faster and more
308 rapid flower differentiation in 'Anna' in comparison with high-chill cultivars was also reported in
309 'Anna' trees grown in warm climate (Oukabli et al. 2003). The present study showed that flower
310 differentiation in 'Anna' was also faster even in cold winter climate region in temperate zone. In
311 contrast, floral differentiation progresses was slower in low-chill cultivars than in high-chill cultivars
312 in *Prunus* species such as peach (Yamane et al. 2011), sweet cherry (*P. avium*) (Fadón et al. 2018), and
313 Japanese apricot (*P. mume*) (Kitamura et al. 2016). One of the possibilities to explain the discrepancy
314 between apple and *Prunus* is that they have different ability to respond to dormancy inductive
315 environmental conditions. Typical apple cultivars do not respond to decreasing photoperiod to induce
316 dormancy (Heide and Prestrud 2005), whereas *Prunus* fruit trees usually respond both decreasing
317 photoperiod and temperature to induce dormancy (Heide 2008). However, more comprehensive
318 microscopic observation using other apple cultivars showing contrasting chilling requirement will be
319 required to confirm the relationship between chilling requirement and flower development in apple.
320
321 MdFLC-like *may prevent the outgrowth of dormant apple flower buds when winter temperatures are*
322 *low*
323 Based on our hypothesis that *MdFLC-like* may function in vegetative growth inhibition, a

possible role of *MdFLC-like* in flower buds during winter could be prevention of unexpected bud outgrowth during late endodormancy and early ecodormancy. *MdFLC-like* expression was down-regulated in response to warm temperatures. High *MdFLC-like* expression level in ecodormant period may, thus, contribute to the heat requirement for bud break of dormant buds. Decreased expression level of *MdFLC-like* in response to warm temperatures towards spring may lead to the actuation of the buds outgrowth competency in spring.

For the phenological growth regulation of perennial plants, growth inhibition in winter could be systemically maintained by particular regulatory network. Expressions of the genes in a monophyletic *FLC* clade in *Arabidopsis* were known to be regulated by cold temperatures (Gu et al. 2013). Furthermore, this cold temperature-dependent regulation of *FLC* expression is known to be mediated by histone modifications of the *FLC* locus in *Arabidopsis* (Bastow et al. 2004). This study showed that *MdFLC-like* appeared to function as a growth regulator in response to cold and warm temperature and also to the flower primordia development. Biological significance of the upregulation of *MdFLC-like* during endodormancy progress and the functional characterization of *MdFLC-like* in *Malus* background would further highlight the significance of *FLC* homologs on the perennial life cycles of Rosaceae plants.

Conclusions

This study proposed the possibility of the regulational conservation of an *FLC* homologs in

Rosid plants, with regards to the temperature sensitivity, although the directions of the expression changes in response to chilling accumulation were opposite between *Arabidopsis* and *Malus*. The gene expression was positively correlated with chilling accumulation in *Malus* and they were negatively correlated in *Arabidopsis*. In addition, the *MdFLC-like* function in growth inhibition is different from that of Brassicaceae *FLC* in flowering repression. Interestingly, the *MdFLC-like* expression appeared to be robustly controlled under fluctuating environmental conditions and was highly associated with the fulfillment of the chilling requirement in different apple genotypes. This suggested the involvement of *MdFLC-like* in the regulatory system underlying the bud dormancy transition and flower developmental regulation during winter. Consequently, we hypothesized that *MdFLC-like* has a role in growth regulation of apple flower buds during late endodormancy and ecodormancy periods to prevent bud outgrowth. The significance of this pathway in the dormancy process of apple should be addressed in the future.

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Conflict of interest

The authors declare that no competing interests exist.

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References

Aikawa S, Kobayashi M, Satake A, Shimizu KK, Kudoh H (2010) Robust control of the seasonal expression of the *Arabidopsis FLC* gene in a fluctuating environment. PNAS 107:11632–11637.

Allard A, Legave JM, Martinez S, Kelner JJ, Bink MCAM, Di Guardo M, Di Pierro EA, Laurens F, van de Weg EW, Costes E (2016) Detecting QTLs and putative candidate genes involved in budbreak and flowering time in an apple multiparental population. J Exp Bot 67:2875–2888.

Bielenberg DG, Rauh B, Fan S, Gasic K, Abbott AG, Reighard GL, Okie WR, Wells CE (2015) Genotyping by sequencing for SNP-based linkage map construction and QTL analysis of chilling requirement and bloom date in peach [*Prunus persica* (L.) Batsch]. PLoS ONE 10:e0139406.

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature 427:164–167.

Brooks RM, Olmo HP (1972) Register of new fruit and nut varieties: second edition, 2 edn. Univ of California Press, Berkeley

Celton J-M, Martinez S, Jammes M-J, Bechti A, Salvi S, Legave J-M, Costes E (2011) Deciphering the genetic determinism of bud phenology in apple progenies: a new insight into chilling and

384 heat requirement effects on flowering dates and positional candidate genes. New Phytol
385 192:378–92.
386 Considine MJ, Considine JA (2016) On the language and physiology of dormancy and quiescence in
387 plants. J Exp Bot 67: 3189–3203.
388 Cooke JK, Eriksson ME, Junttila, O (2012) The dynamic nature of bud dormancy in trees:
389 environmental control and molecular mechanisms. Plant Cell Env 35:1707–1728.
390 Daccord N, Celton J-M, Linsmith G, Becker C, Choisne N, Schijlen E, van de Geest H, Bianco L,
391 Micheletti D, Velasco R, Di Pierro EA, Gouzy J, Rees DJG, Guérif P, Muranty H, Durel C-E,
392 Laurens F, Lespinasse Y, Gaillard S, Aubourg S, Quesneville H, Weigel D, van de Weg E,
393 Troggio M, Bucher E (2017) High-quality de novo assembly of the apple genome and
394 methylome dynamics of early fruit development. Nat Genet 49:1099–1106.
395 Deng W, Ying H, Helliwell CA, Taylor JM, Peacock WJ, Dennis ES (2011) FLOWERING LOCUS
396 C (FLC) regulates development pathways throughout the life cycle of *Arabidopsis*. PNAS
397 108:6680–6685.
398 Doğramacı M, Horvath DP, Anderson JV (2014) Dehydration-induced endodormancy in crown buds
399 of leafy spurge highlights involvement of MAF3-and RVE1-like homologs, and hormone
400 signaling cross-talk. Plant Mol Biol 86:409-424.
401 Du X, Xiao Q, Zhao R, Wu F, Xu Q, Chong K, Meng Z (2008) *TrMADS3*, a new MADS-box gene,
402 from a perennial species *Taihangia rupestris* (Rosaceae) is upregulated by cold and experiences
403 seasonal fluctuation in expression level. Dev Genes Evol 218:281–92.

van Dyk MM, Soeker MK, Labuschagne IF, Rees DJG (2010) Identification of a major QTL for time of initial vegetative budbreak in apple (*Malus* x *domestica* Borkh.). Tree Genet Genomes 6:489–502.

Fadón E, Rodrigo J, Herrero M (2018) Is there a specific starge to rest? Morphological changes in flower primordia in relation to endodormancy in sweet cherry (*Prunus avium* L.). Trees 32:1583–1594.

Falavigna VS, Guitton B, Costes E, Andres F (2019) I want to (Bud) break free: The potential role of *DAM* and *SVP*-like genes in regulating dormancy cycle in temperate fruit trees. Front Plant Sci 9:1990

Gu X, Le C, Wang Y, Li Z, Jiang D, Wang Y, He Y (2013) *Arabidopsis* FLC clade members form flowering-repressor complexes coordinating responses to endogenous and environmental cues. Nat Commun 4:1947.

Heide OM. (2008) Interaction of photoperiod and temperature in the control of growth and dormancy of *Prunus* species. Sci Hort 115:309-314.

Heide OM, Prestrud AK (2005) Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol 25:109–14.

Imai T, Ubi BE, Saito T, Moriguchi T (2014) Evaluation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in *Pyrus pyrifolia* using different tissue samples and seasonal conditions. PLoS One 9:e86492.

Kawamoto T (2003) Use of a new adhesive film for the preparation of multi-purpose fresh-frozen

424 sections from hard tissues, whole-animals, insects and plants. Arch Histol Cytol 66:123–143
425 Kitamura Y, Takeuchi T, Yamane H, Tao R (2016) Simultaneous down-regulation of *DORMANCY-*
426 *ASSOCIATED MADS-box6* and *SOC1* during dormancy release in Japanese apricot (*Prunus*
427 *mume*) flower buds. J Hort Sci Biotech 91:476–482
428 Kitamura Y, Habu T, Yamane H, Nishiyama S, Kajita K, Sobue T, Kawai T, Numaguchi K, Nakazaki
429 T, Kitajima A, Tao R (2018) Identification of QTLs controlling chilling and heat requirements
430 for dormancy release and bud break in Japanese apricot (*Prunus mume*). Tree Gen Genome 14:33
431 Kumar G, Arya P, Gupta K, Randhawa V, Acharya V, Singh AK (2016) Comparative phylogenetic
432 analysis and transcriptional profiling of MADS-box gene family identified *DAM* and *FLC*-like
433 genes in apple (*Malus* x *domestica*). Sci Rep 6:20695.
434 Kurokura, T, Mimida N, Battey NH, Hytönen T (2013) The regulation of seasonal flowering in the
435 Rosaceae. J Exp Bot 64:4131-4141.
436 Kotoda N, Hayashi H, Suzuki M, Igarashi M, Hatsuyama Y, Kidou SI, Igasaki T, Nishiguchi M, Yano
437 K, Shimizu T, Takahashi S, Iwanami H, Moriya S, Abe K. (2010). Molecular characterization
438 of *FLOWERING LOCUS T*-like genes of apple (*Malus*× *domestica* Borkh.). Plant Cell Physiol
439 51: 561–575.
440 Lang GA (1987) Dormancy : A new universal terminology. HortSci 25:817–820.
441 Lang GA, Early JD, Martin GC, Darnell RL (1987) Endo-, para-, and ecodormancy: physiological
442 terminology and classification for dormancy research. HortSci 22:371–377.
443 Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein

444 that acts as a repressor of flowering. Plant Cell 11:949–56.

445 Miotto EV, Tessele C, Czermainski ABC, Porto DD, Falavigna VS, Sartor T, Cattani AM, Delatorre
446 CA, de Alencar SA, da Silva-Jnior OB, Togawa RC, Costa MMC, Pappas Jr GJ, Grynberg P, de
447 Oliveira PRD, Kvitschal MV, Denardi F, Buffon V, Revers LF (2019) Spring is coming: Genetic
448 analyses of the bud break date locus reveal candidate genes from the cold perception pathway to
449 dormancy release in apple (*Malus* × *domestica* Borkh.) Front. Plant Sci 10:33.

450 Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo
451 T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing
452 efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104:34–41.

453 Oukabli A, Bartolini S, Viti R (2003) Anatomical and morphological study of apple (*Malus*×
454 *domestica* Borkh.) flower buds growing under inadequate winter chilling. J Hort Sci Biotech
455 78:580–585.

456 Porto DD, Bruneau M, Perini P, Anzanello R, Renou J-P, dos Santos HP, Fialho FB, Revers LF (2015)
457 Transcription profiling of the chilling requirement for bud break in apples: a putative role for
458 *FLC*-like genes. J Exp Bot 66:2659–2672.

459 Ratcliffe OJ, Nadzan GC, Reuber TL, Riechmann JL (2001) Regulation of flowering in *Arabidopsis*
460 by an *FLC* homologue. Plant Physiol 126:122–32.

461 Reinoso H, Luna V, Pharis RP, Bottini R (2002) Dormancy in peach (*Prunus persica*) flower buds. V.
462 Anatomy of bud development in relation to phenological stage. Can J Bot 80:656–663.

463 Richardson E, Seeley S, Walker D (1974) A model for estimating the completion of rest for 'Redhaven'

464 and ‘Elberta’ peach trees. HortSci 9:331–332.
465 Saito T, Wang S, Ohkawa K, Ohara H, Ikeura H, Ogawa Y, Kondo S (2017) Lipid droplet-associated
466 gene expression and chromatin remodelling in *LIPASE* 5-upstream region from beginning-to
467 mid-endodormant bud in‘Fuji’apple. Plant Mol Biol 95:441–449
468 Searle I, He Y, Turck F, Vincent C, Fornara F, Kröber S, Amasino RA, Coupland G (2006) The
469 transcription factor FLC confers a flowering response to vernalization by repressing meristem
470 competence and systemic signaling in *Arabidopsis*. Genes Dev 20:898–912.
471 Takeuchi T, Matsushita MC, Nishiyama S, Yamane H, Banno K, Tao R (2018) RNA-sequencing
472 analysis identifies genes associated with chilling-mediated rndodormancy release in apple. J Am
473 Soc Hortic Sci 143:194–206.
474 Trainin T, Zohar M, Shimoni-Shor E, Doron-Faigenboim A, Bar-Ya'akov I, Hatib K, Sela N, Holland
475 D, Isaacson T (2016) A unique haplotype found in apple accessions exhibiting early bud-break
476 could serve as a marker for breeding apples with low chilling requirements. Mol Breed 36:158
477 Urrestarazu J, Muranty H, Denancé C, Leforestier D, Ravon E, Guyader A, Guisnel R, Feugey L,
478 Aubourg S, Celton J-M, Daccord N, Dondini L, Gregori R, Lateur M, Houben P, Ordidge M,
479 Paprstein F, Sedlak J, Nybom H, Garkava-Gustavsson L, Troggio M, Bianco L, Velasco R,
480 Poncet C, Théron A, Moriya S, Bink MCAM, Laurens F, Tartarini S, Dure CE. (2017) Genome-
481 wide association mapping of flowering and ripening periods in apple. Front Plant Sci 8:1923
482 Wang R, Farrona S, Vincent C, Joecker A, Schoof H, Turck F, Alonso-Blanco C, Coupland G, Albani
483 MC (2009) *PEP1* regulates perennial flowering in *Arabis alpina*. Nature 459:423–427.

Wang SY, Jiao HJ, Faust M (1991) Changes in superoxide dismutase activity during thidiazuron-induced lateral budbreak of apple. HortSci 26:171–173.

Wisniewski M, Norelli J, Artlip T (2015) Overexpression of a peach CBF gene in apple: a model for understanding the integration of growth, dormancy, and cold hardiness in woody plants. Front Plant Sci 6:85.

Yamane H (2014) Regulation of bud dormancy and bud break in Japanese apricot (*Prunus mume* Siebold & Zucc.) and peach [*Prunus persica* (L.) Batsch]: A summary of recent studies. J Japan Soc Hort Sci 83:187–202.

Yamane H, Ooka T, Jotatsu H, Sasaki R, Tao R (2011) Expression analysis of *PpDAM5* and *PpDAM6* during flower bud development in peach (*Prunus persica*). Sci Hort 129:844–848.

Zhang X, Henriques R, Lin S-S, Niu Q-W, Chua N-H (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1:641–646.

Zhu Y, Li Y, Xin D, Chen W, Shao X, Wang Y, Guo W (2015) RNA-Seq-based transcriptome analysis of dormant flower buds of Chinese cherry (Prunus pseudocerasus). Gene 555:362–376.

501 **Figure Legends**

502

503 **Figure 1.** Characterization of *MdFLC-like*-overexpressing *Arabidopsis*. Bar plots of (A) the number
504 of days to bolting; (B) number of days to flowering, (C) number of rosette leaves at bolting, (D)
505 number of rosette leaves at flowering for *35S:MdFLC-like* and wild type (WT) plants. In total, 10
506 independent transgenic lines were monitored for each genotype. Mean values are indicated as "x".
507 Significant differences were determined using a t-test, and p-values were designated.

508

509 **Figure 2.** Typical appearance of *MdFLC-like*-overexpressing *Arabidopsis*. (A, B) Left: wild type
510 (WT), right: 35S:*MdFLC-like*; (C) WT; (D, E) *35S:MdFLC-like*. Arrows indicate developed scapes.

511

512 **Figure 3.** Characterization of seasonal changes in the dormancy states of different apple cultivars.
513 Bars and lines indicate the bud burst rate and the average number of weeks required for the bud burst
514 under forcing conditions, respectively. At least five shoots were assessed at each sampling date. (A)
515 'Fuji' in 2016–2017, (B) 'Anna' in 2016–2017, (C) 'Tsugaru' in 2017–2018, (D) 'Anna' in 2017–2018.

516

517 **Figure 4.** Expression patterns of *MdFLC*-like in apple terminal flower buds (A) in the 2016−2017 and
518 (B) 2017−2018 seasons. Error bars indicate the standard errors for three to five biological replicates.

519

520 **Figure 5.** Effects of warm temperature on *MdFLC*-like expression in apple ecodormant flower buds.

Shoots bearing flower buds collected at 1200CU (left) were treated with warm temperatures (right). Error bars indicate the standard errors of three biological replicates.

Figure 6. Microscopic observations of flower meristem development in dormant apple buds during dormancy. At least five flower buds were observed at each sampling, and representative tissue sections are shown. (A) Overall picture of longitudinal sections of terminal flower bud; and (B) Enlarged picture of central and lateral flower primordia of longitudinal sections of different terminal flower buds collected at the same date as that in (A). All pictures in (B) were under the same scale.

Supplementary Figure 1. Amino acid sequence alignment of FLC homologs of Arabidopsis and apple identified by Takeuchi et al., 2018.

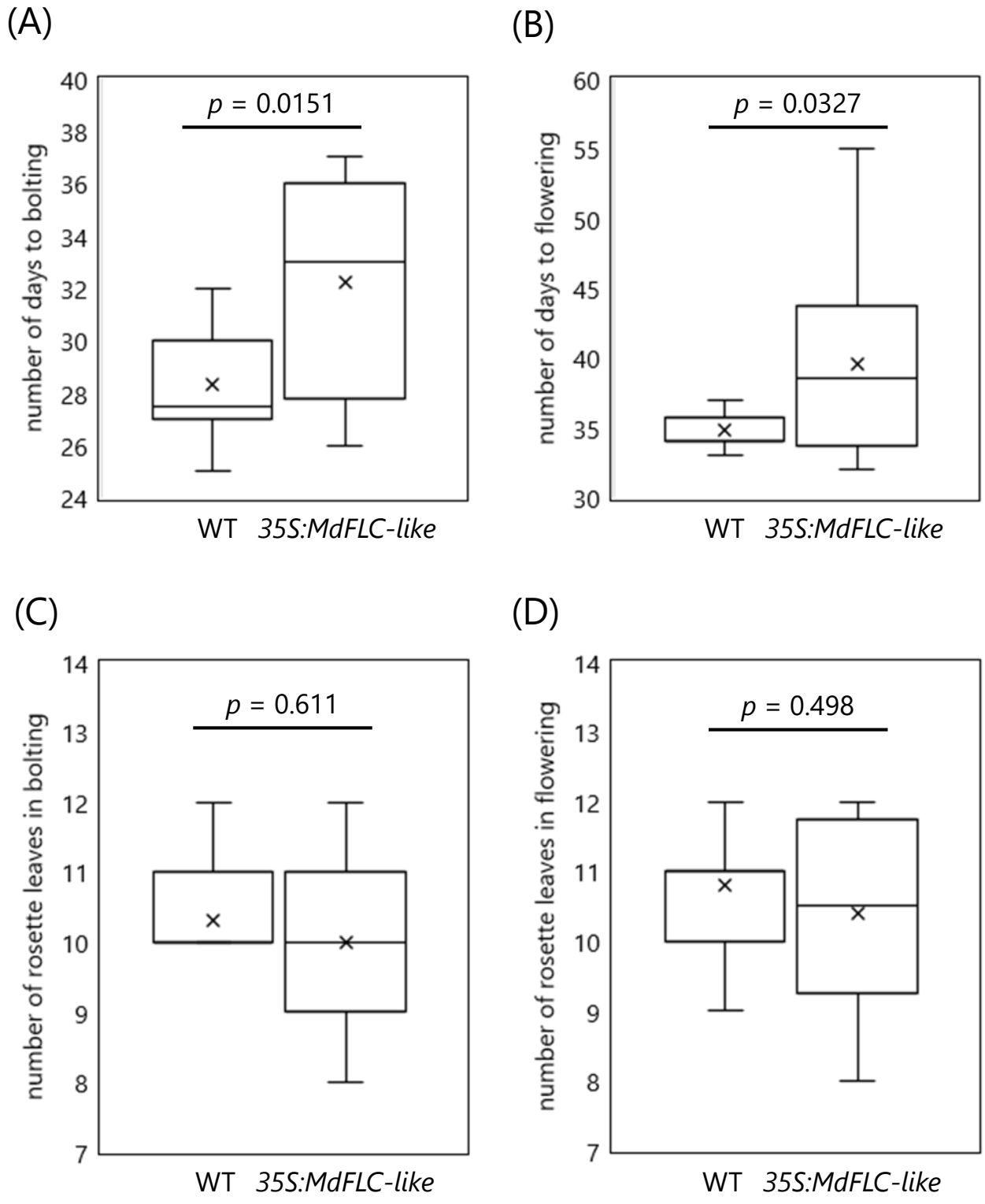

(A)
number of days to bolting
40
38
36
34
32
30
28
26
24
p = 0.0151
WT
35S:MdFLC-like
(B)
number of days to flowering
60
55
50
45
40
35
30
p = 0.0327
WT
35S:MdFLC-like
(C)
number of rosette leaves in bolting
14
13
12
11
10
9
8
7
p = 0.611
WT
35S:MdFLC-like
(D)
number of rosette leaves in flowering
14
13
12
11
10
9
8
7
p = 0.498
WT
35S:MdFLC-like

Figure 2

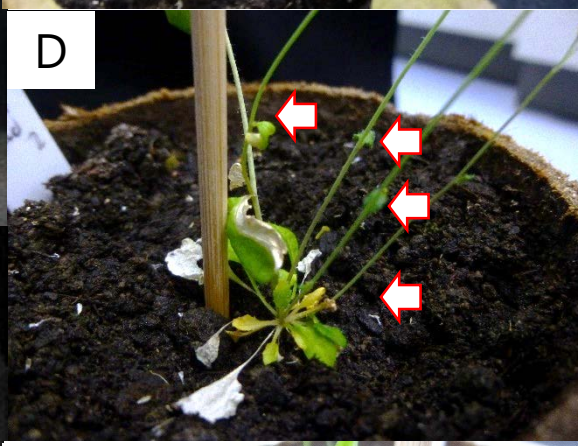


Figure 3

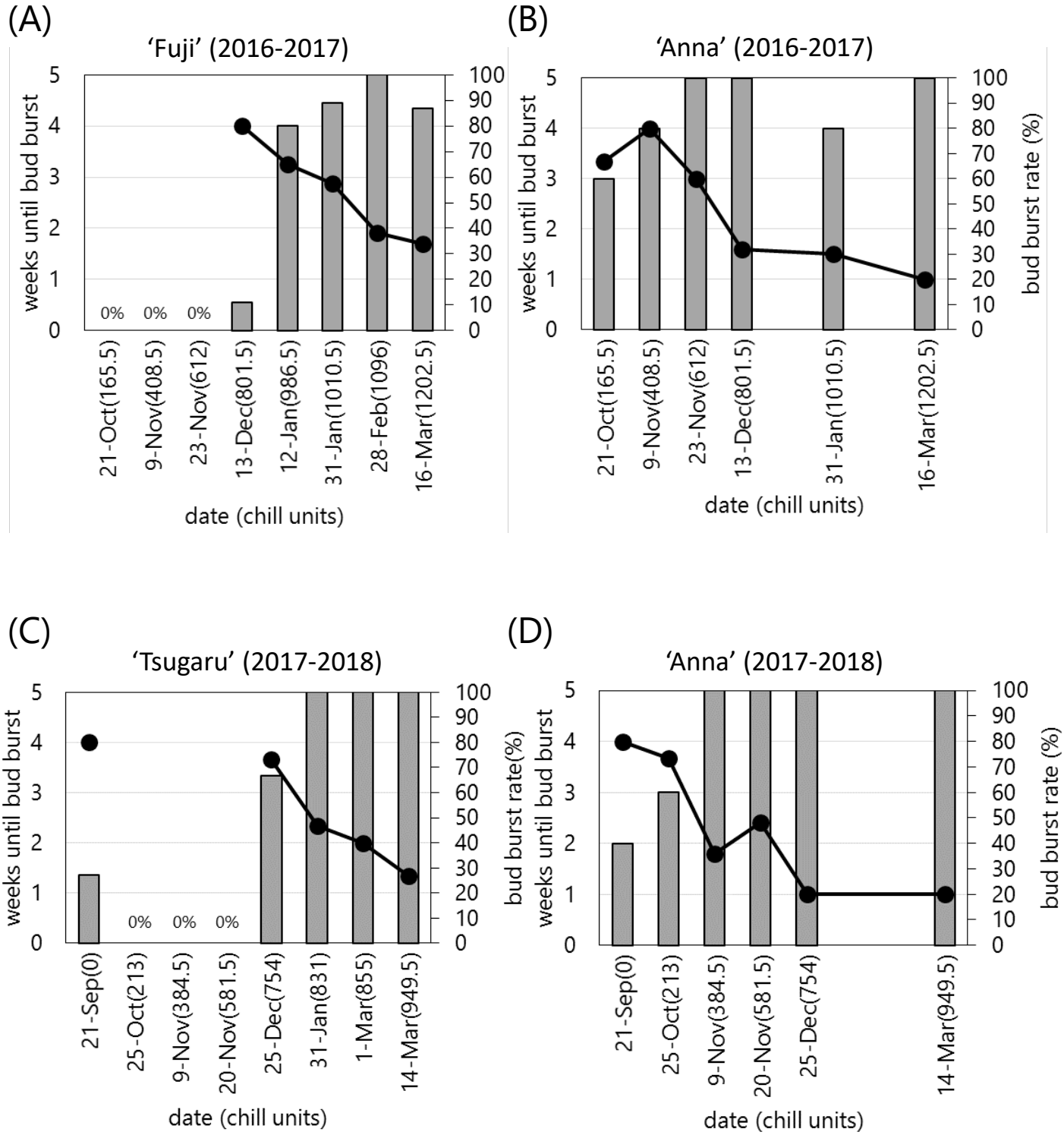
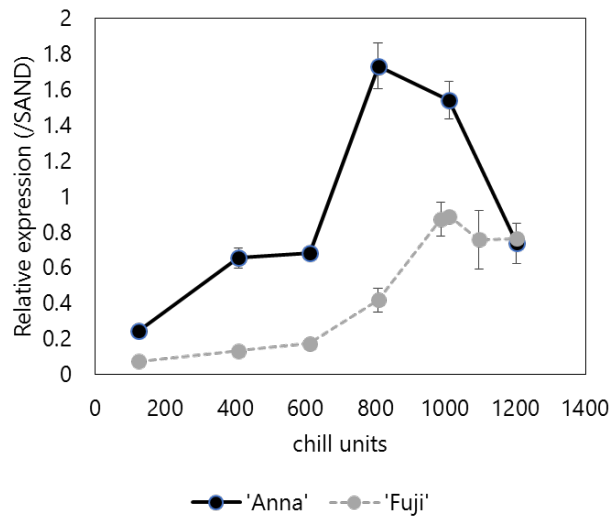

(A)
'Fuji' (2016-2017)
weeks until bud burst
bud burst rate (%)
0%
0%
0%
21-Oct(165.5)
9-Nov(408.5)
23-Nov(612)
13-Dec(801.5)
12-Jan(986.5)
31-Jan(1010.5)
28-Feb(1096)
16-Mar(1202.5)
date (chill units)
(B)
'Anna' (2016-2017)
weeks until bud burst
bud burst rate (%)
21-Oct(165.5)
9-Nov(408.5)
23-Nov(612)
13-Dec(801.5)
31-Jan(1010.5)
16-Mar(1202.5)
date (chill units)
(C)
'Tsugaru' (2017-2018)
weeks until bud burst
bud burst rate (%)
0%
0%
0%
21-Sep(0)
25-Oct(213)
9-Nov(384.5)
20-Nov(581.5)
25-Dec(754)
31-Jan(831)
1-Mar(855)
14-Mar(949.5)
date (chill units)
(D)
'Anna' (2017-2018)
bud burst rate(%)
weeks until bud burst
bud burst rate (%)
21-Sep(0)
25-Oct(213)
9-Nov(384.5)
20-Nov(581.5)
25-Dec(754)
14-Mar(949.5)
date (chill units)

Figure 4

(A)


Relative expression (/SAND)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
1200
1400
chill units
'Anna'
'Fuji'

(B)

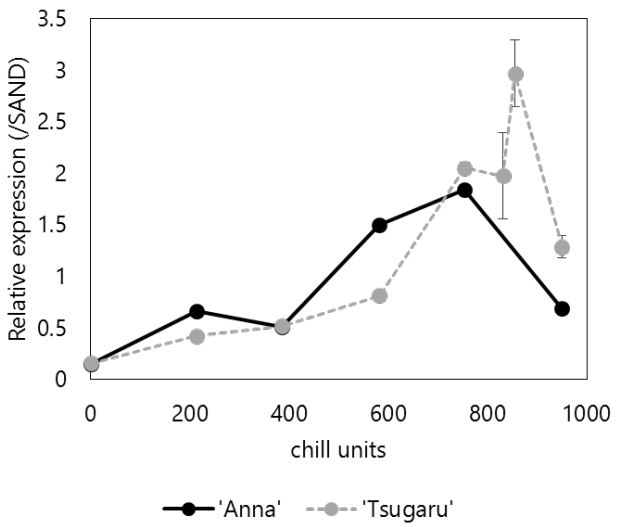

Relative expression (/SAND)
3.5
3
2.5
2
1.5
1
0.5
0
0
200
400
600
800
1000
chill units
'Anna'
'Tsugaru'

Figure 5

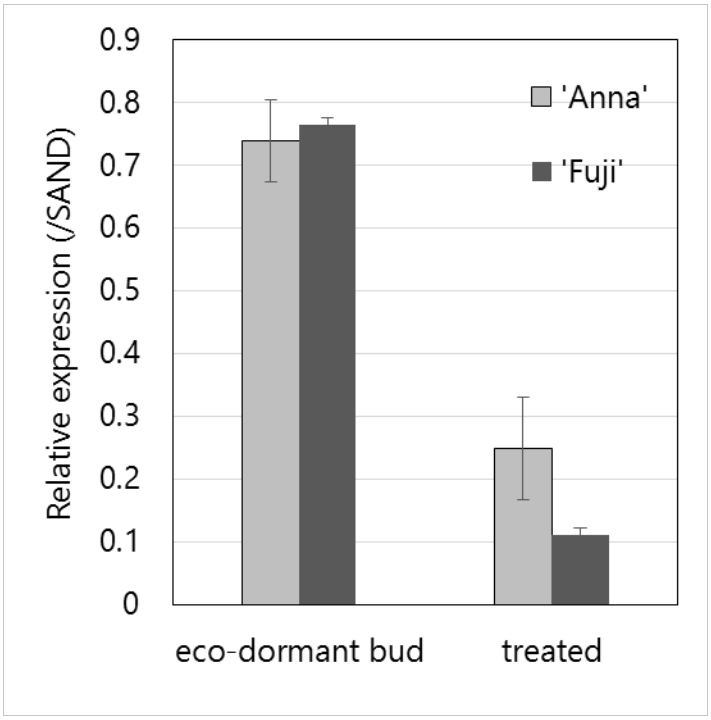
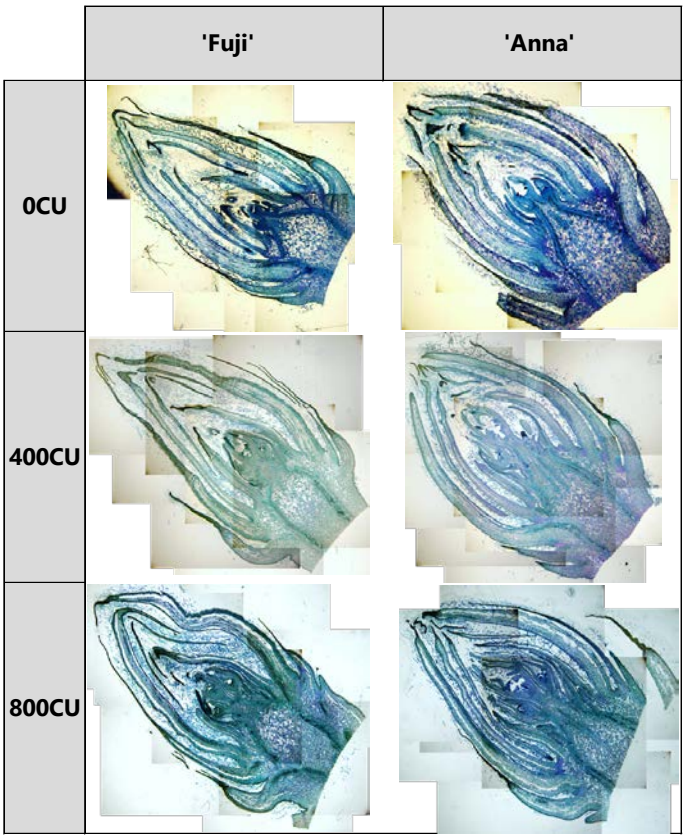
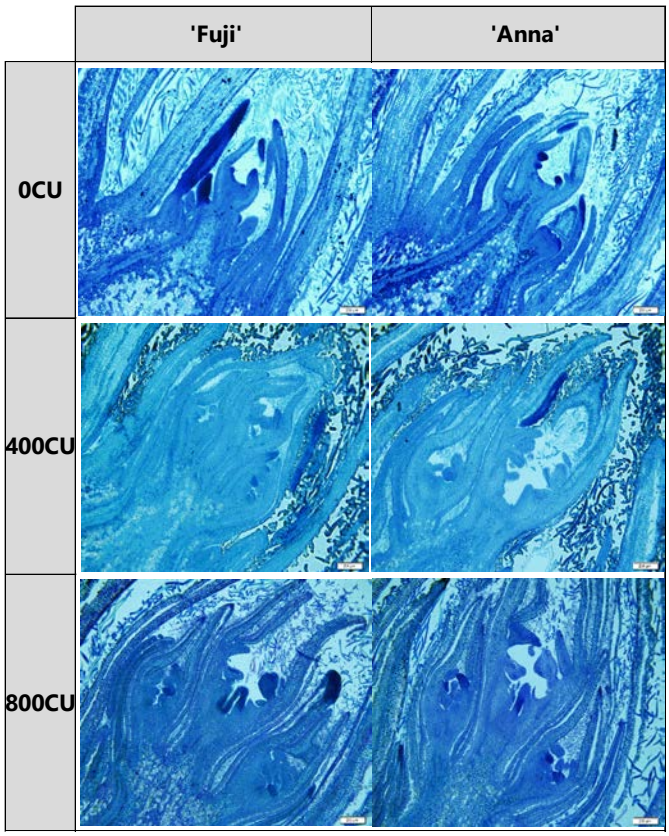

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative expression (/SAND)
'Anna'
'Fuji'
eco-dormant bud
treated

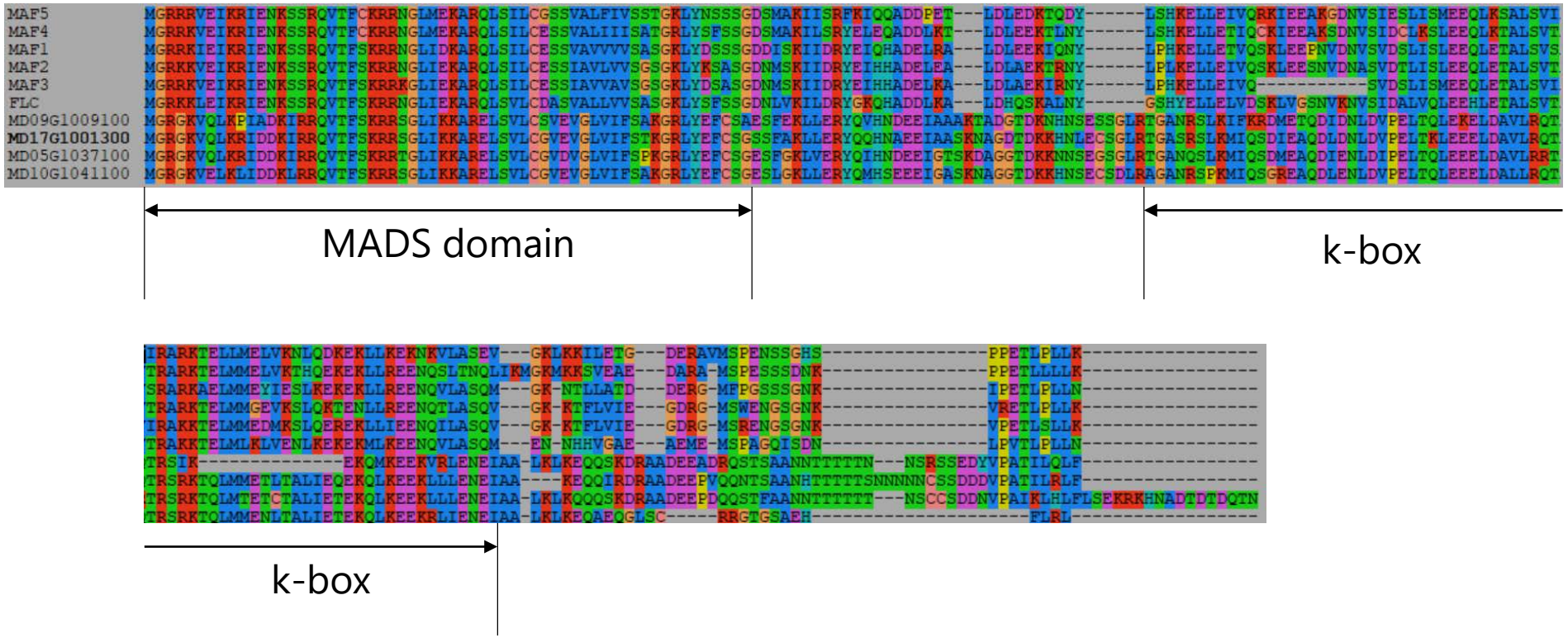
Figure 6

(A)



(B)





Supplementary Figure 1
Amino acid sequence alignment of FLC homologs of Arabidopsis and apple identified by Takeuchi et al., 2018.