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Research paper

# Physiological differences between bud breaking and flowering after dormancy completion revealed by *DAM* and *FT/TFL1* expression in Japanese pear (*Pyrus pyrifolia*)

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**The regulatory mechanisms underlying bud breaking (scale leaf elongation) and flowering in the lateral flower buds of Japanese pear (*Pyrus pyrifolia* Nakai 'Kosui') are unknown. To more fully characterize these processes, we treated pear trees with different amounts of chilling initiated at different times. Chilling for ~900 h at 6 °C always induced bud breaking (scale elongation in ≥70% lateral flower bud) when provided between October and February, whereas chilling provided earlier (between October and December) was less effective on flowering (floret growth and development) than later chilling and the flowering rate increased with longer chilling durations. During chilling, the expression of pear *DAMs* (*PpMADS13-1*, *13-2* and *13-3*) in lateral flower buds decreased as chilling accumulated irrespective of the timing of chilling. In addition, pear *TFL1* (*PpTFL1-1a*) in the lateral flower buds was expressed at higher levels when the time interval for chilling was earlier. On the other hand, during forcing at 15 °C after chilling, the expression pattern of all three *PpMADS13* genes was similar among the treatments, and the expression levels seemed lower in the treatment where scale leaves of the lateral flower bud elongated faster, whereas pear *FT* (*PpFT2a*) was expressed at higher levels in the buds whose flower clusters elongated more vigorously during forcing. From these results, we infer that flowering time may be mediated via the balance of flowering-related genes *FT* and *TFL1*, whereas bud breaking may be regulated via the *DAM* genes in Japanese pear.**

***Keywords***: bloom, bud scale elongation, chill requirement.

## Introduction

Perennial woody plants adapt to seasonal environmental changes by modulating their growth rhythm, among which seasonal growth cessation is an important strategy for survival during cold winters (Cooke et al. 2012). Growth cessation during the winter season is a well-studied phenomenon, known as bud dormancy, and is divided into three physiological phases: paradormancy, endodormancy and ecodormancy (Lang 1987, Anderson et al. 2005). Endodormancy is the state in which bud growth is arrested not by external factors but by internal factors. Once endodormancy is established, buds are incapable of resuming their growth until a chilling requirement is satisfied, the amount of which is genetically determined. Endodormancy is induced in response to short days in many tree species (reviewed by Horvath 2009), whereas for plants in the Pyroideae subfamily, such as Japanese pear (*Pyrus pyrifolia* Nakai) (Takemura et al. 2011), European pear (*P. communis* L.) and apple (*Malus* × *domestica* Borkh.) (Heide and Prestrud 2005), photoperiod does not influence the induction and progression of endodormancy.

The molecular regulation of endodormancy has been extensively studied in woody plants. *Dormancy-associated MADS-box* (*DAM*) genes were first identified as candidate genes for

terminal bud formation in the *EVERGROWING* (*EVG*) locus of peach (Bielenberg et al. 2008). Close relationships between the expression of these genes and endodormancy phase transition have been reported (Horvath 2009, Ubi et al. 2010, Yamane et al. 2011). On the other hand, the *CONSTANS* (*CO*)/*Flowering Locus T* (*FT*) module, a well-known component playing a critical role in flowering induction, is reported to play an important role for winter bud dormancy in the transition from the active to the endodormancy phase in photoperiod-dependent plants such as *Populus* species (Böhlenius et al. 2006, Hsu et al. 2011, Rinne et al. 2011) and *Vitis vinifera* L. (Pérez et al. 2011). Terminal Flower 1 (TFL1) is an antagonist of FT in flowering regulation and competes with FT for FLOWERING LOCUS D (FD) binding (Hanano and Goto 2011). Overexpression of *TFL1* alters chilling requirements and delayed bud burst in *Populus tremula* L. × *P. alba* L. (Mohamed et al. 2010). Dormancy progression in woody perennial plants is tightly regulated probably via molecular networks where the genes, including those mentioned above, may be important players in harmony with environmental changes to survive severe conditions during winter. In pear trees, however, the roles of the CO/FT module components and their relationship with TFL1 in the regulation of endodormancy transition have not yet been elucidated.

Japanese pear (Japanese common name, Nashi) is one of the most important fruits grown in Japan, with production in 2012 at 267,200 tons. Japanese pear is grown in subtropical to temperate climate areas in Japan. Flowering is an important event that greatly influences the economy of fruit tree production. Japanese pear flower buds contain both floral primordia and vegetative primordia (mixed flower buds), with the number of primordia varying genetically and environmentally. Flower buds are usually the terminal buds on spurs and terminal and lateral buds on long shoots. Floral induction of Japanese pear trees occurs in late summer, and floral organs develop until autumn. Floral buds enter into endodormancy in the autumn, and flower in the next spring after passing through winter. Flower buds become distinguishable from vegetative buds as the former appears larger than the latter when leaves are shedding in the autumn. In our previous study, we exposed Japanese pear 'Kosui' potted trees to various temperatures for different durations and determined that temperatures between −6 and 12 °C have positive effects on dormancy progression (Sugiura and Honjo 1997). The most effective temperature range for chilling is between 0 and 6 °C for a 750-h exposure to induce sufficient bud break and subsequent flowering (endodormancy release).

In this study, we intended to uncover the different regulatory mechanisms underlying bud breaking and flowering and to show the respective roles of DAMs and the CO/FT module in the regulation of Japanese pear dormancy. For this purpose, we treated pear trees with varying amounts of chilling at different times during the autumn–winter months, and induced bud breaking (scale leaf elongation) and flowering separately. Consequently, we conducted gene expression analyses in these trees and identified possibly different roles for DAMs and the CO/FT module in these developmental processes.

## Materials and methods

### Plant materials

This study was undertaken in the experimental orchard of the NARO Institute of Fruit Tree Science, Tsukuba, Japan (36°N, 140°E) during the winter months of 2012–13 and 2013–14. Potted trees of Japanese pear (*P. pyrifolia* 'Kosui', 2 or 3 years old) grafted on *P. calleryana* Decne. were used for experiments.

### Experimental plots

In all experiments, trees were subjected to one of the following three conditions: field conditions (natural temperature/photoperiod), 6 °C in an incubator (dark) or 15 °C in a phytotron with a natural photoperiod. In pear trees, low temperature, but not photoperiod, is a prerequisite for the progression of endodormancy (Heide and Prestrud 2005, Takemura et al. 2011). Therefore, photoperiod was not considered important when we subjected trees to 6 °C treatments.

### Dormancy evaluation

In this study, endodormancy release was defined as occurring when ≥70% of the lateral flower buds of a plant flowered during 2 months of forcing treatment (15 °C, natural photoperiod). Each Japanese pear flower bud (Figure 1a) usually contains several flowers (florets), and a flower bud has 'flowered' when one or more floret(s) appear from each flower bud (Figure 1c and d).

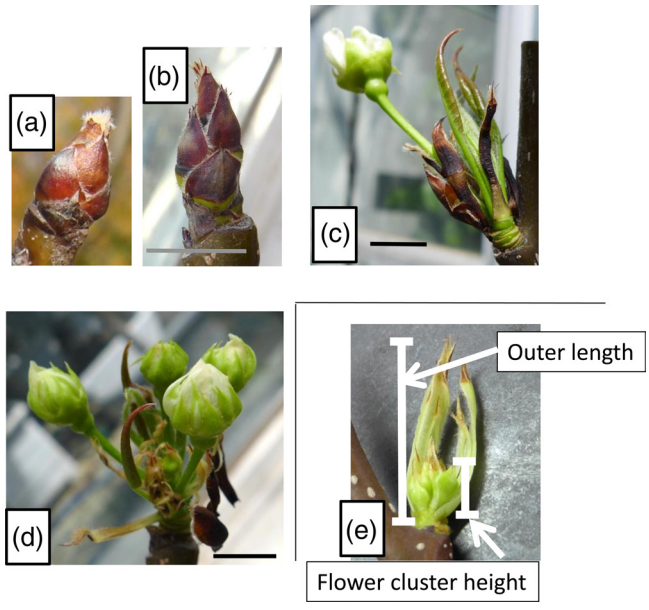


Figure 1. Japanese pear flower bud morphology: (a) dormant, (b) bud breaking (base green), (c and d) flowering, (e) inner of scale leaves showing definitions of bud and flower cluster parameters measured for Figure 4. Scale bars, 1 cm (b–d).

We have already shown that temperatures between −6 and 12 °C have positive effects on endodormancy release for the lateral flower buds of 'Kosui', and the most effective temperature range is between 0 and 6 °C with a 750-h exposure to these temperature regimes required for sufficiently releasing endodormancy (Sugiura and Honjo 1997). We treated trees at 6 °C for 600, 750, 900, 1200, 1500 and 1800 h at chilling temperatures, and these are 0.8, 1.0, 1.2, 1.6, 2.0 and 2.4 times, respectively, the theoretically required amount of chilling for endodormancy release according to our previous report (Sugiura and Honjo 1997). On the other hand, when trees accumulated chilling in field conditions, we calculated the chilling amount according to the chill unit model we developed previously for 'Kosui' lateral flower buds (DeVelopmental Index (DVI) model; Sugiura and Honjo 1997) and report these data as a proportion of the theoretically required amount of chilling for endodormancy release (e.g., DVI = 1.0 when trees accumulated 1.0 times the theoretically required amount of chilling for endodormancy release).

Air temperatures in the controlled environments were monitored with thermo recorders (TR-71U, T&D Corporation, Tokyo, Japan), and those in the field conditions (hourly mean temperature) were obtained from the nearest weather station (Japan Meteorological Agency, Tsukuba, Ibaraki Japan) located ~1.5 km west of the experimental orchard.

### *Chilling accumulation in an incubator*

***2012–13*** To follow endodormancy progression and the encountering of low temperatures, potted 'Kosui' trees were treated at 6 °C in the dark for ~600, 900 and 1200 h (corresponding to 0.8, 1.2 and 1.6 times sufficient chilling for endodormancy release, respectively) at different starting times, i.e., on 1 November or 3 December in 2012, or on 4 January or 1 February in 2013 (Figure 2). One exception to the protocol was made; for trees chilled starting on 1 November, 1125 h of chilling, not 1200 h, was provided which corresponds to 1.5 times sufficient chilling for endodormancy release. In order to avoid chilling, trees were moved from the field into 15 °C conditions on 1 November (trees had accumulated natural chilling (<6 °C) <10 h until 1 November) and incubated until the chilling was initiated. After trees accumulated their corresponding chilling duration, lateral flower buds of one shoot (70–100 cm) from each of the two trees was excised for mRNA analyses, and then trees that had received 600 or 900 h of chilling were moved into 15 °C conditions again and forced for 2 months. Because of the space limitations for temperature treatments at 6 and 15 °C, we used only two potted trees for each treatment and a total of six trees for each chilling interval. For comparison, we also forced trees to accumulate chilling for DVI = 0.8, 1.0 and 1.2 in the field conditions and compared the flowering rate with trees that received artificial chilling. In 2012, pear tree leaf senescence progressed in a manner similar to other years; in the natural (field) condition, leaf shedding began at approximately 20 November and was almost completed by the beginning of December.

***2013–14*** We repeated the experiments conducted in 2012–13 in order to confirm the results, but the chilling duration was changed to 600, 900 and 1200 h, or longer when the trees flowered insufficiently with these chilling durations. The procedures for the treatments and samplings are illustrated in Figure 3. Chilling treatment of 6 °C in the dark was initiated on 7 October, 1 November or 4 December in 2013. For a comparison, we also initiated chilling on 31 January but only until 10 March (900 h of 6 °C). After trees accumulated their corresponding chilling

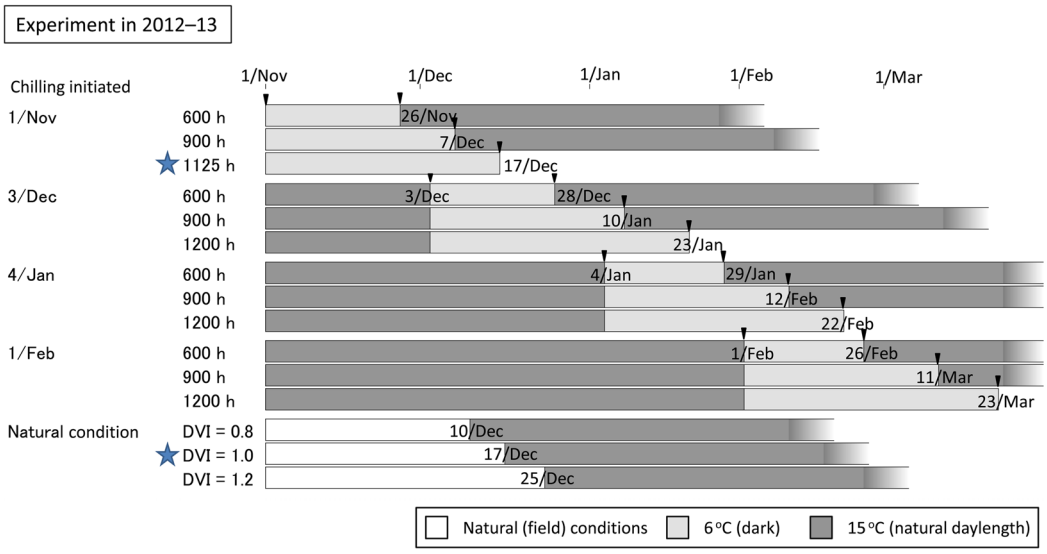


Figure 2. Schematic drawings of chilling treatments conducted in 2012–13. The two rows indicated with stars show the temperature treatments for Figure 4. The dates for lateral flower bud sampling for qPCR are indicated with black arrowheads.

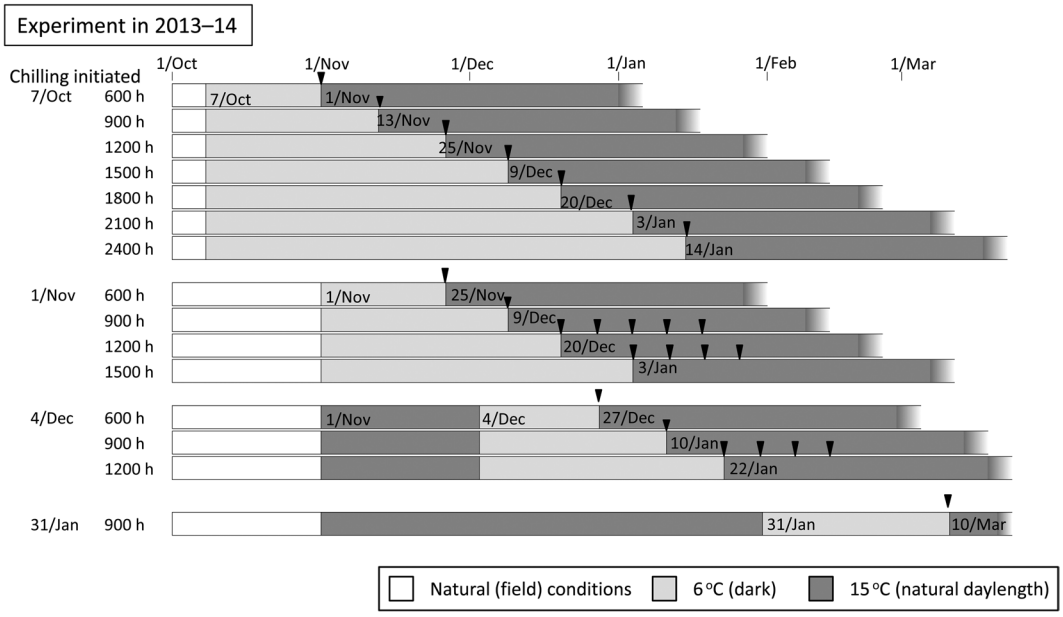


Figure 3. Schematic drawings of chilling treatments conducted in 2013–14. The dates for lateral flower bud sampling for qPCR are indicated with black arrowheads.

duration, lateral flower buds of one shoot (70–100 cm) from each of the two trees were excised for mRNA analyses, and then potted trees were moved into 15 °C and forced for 2 months. We used 2 trees for each sampling and a total of 14, 8, 6 and 2 trees for chilling treatments initiated on 7 October, 1 November, 4 December and 31 January, respectively.

For comparing the growth of flower buds during forcing, the number of flower buds that developed into the bud breaking stage (Figure 1b) and those that had proceeded into flowering (Figure 1c and d) were counted. In addition, for the trees in which chilling was initiated on 1 November for 1200 and 1500 h and those chilled beginning on 4 December for 1200 h, the outer length of the flower bud and the flower cluster height (Figure 1e) were measured during forcing at ~7-day intervals.

### *Total RNA extraction and reverse transcription quantitative polymerase chain reaction*

Total RNA was extracted from lateral flower buds of 'Kosui' according to the method described by Ito et al. (2014). First-strand cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Five microgram aliquots of total RNA used in the reaction were first treated with RNA-free DNaseI (Promega, Madison, WI, USA) and reverse-transcribed using SuperScript III oligo (dT) 20 primers according to the manufacturer's instructions (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a SYBR Premix Ex Taq kit (TaKaRa, Kyoto, Japan) as described in the manufacturer's protocol.

*HistoneH3* and *SAND* were used as internal references in all experiments as reported in Imai et al. (2014). Specific primers for *PpFT1a*, *PpFT2a*, *PpTFL1-1a*, *PpTFL1-2a*, *HistoneH3* and *SAND* were reported in Ito et al. (2014) and those for *PpMADS13-1*, *PpMADS13-2* and *PpMADS13-3* were reported in Saito et al. (2013). qPCR was performed twice using differently extracted total RNA (two biological replications) and each replicate was tested twice (technical replicates) for a total of four measures of expression for each gene. Since the expression trends were similar irrespective of the reference genes used, one dataset for each biological replication using *HistoneH3* was used and averaged to produce the figures.

## Results

### *Flowering and bud breaking (scale leaf elongation) with different timings/amounts of chilling*

In 2012–13, we compared the flowering rate with the same amount of chilling (600 or 900 h), but the treatments were applied at different time intervals. When 600-h chilling was provided, trees did not flower irrespective of when chilling was imposed (Table 1). On the contrary, when chilling was initiated on 1 November, 900-h chilling did not induce flowering, but the flowering rate increased as the initiation of the chilling treatment was delayed and became >70% when the chilling treatment was initiated either on 4 January or 1 February (Table 1). These results suggest that 600-h chilling is insufficient for endodormancy release in Japanese pear lateral flower buds, and that providing the chilling earlier may have no (1 November) or only a small (1 December) impact on

endodormancy release to flowering compared with that provided later (January–February).

Interestingly, the buds exposed to chilling starting on 1 November did not flower during forcing, but their scale leaves elongated and seemed to be undergoing 'bud breaking' (Figure 1b). When buds exposed to chilling in the dark from 1 November to 17 December (1125 h of chilling) were compared with those accumulating chilling under field conditions until the chilling requirement was satisfied for endodormancy release (DVI = 1.0, until 17 December), the scale leaves were longer, but the flower clusters were smaller in the former than the latter after a month of forcing (Figure 4).

Table 1. Effect of different time intervals for starting chilling treatments on the flowering rate (%) of lateral flower buds. Chilling was initiated in November, December, January or February in 2012–13, and the chilling durations were equal (at 6 °C for 600 h as DVI = 0.8, and 6 °C for 900 h as DVI = 1.2, respectively). Different letters indicate a significant difference within a column with Tukey–Krammer's multiple range test ($P < 0.05$) after the data were angularly transformed.

| Timing of chilling initiated | Amount of chilling | |
|---|---|---|
| | 600 h/DVI = 0.8 | 900 h/DVI = 1.2 |
| 1 November | 0 | 0 a |
| 3 December | 0 | 34.1 b |
| 4 January | 0 | 79.1 c |
| 1 February | 0 | 85.7 c |
| Natural condition (chilling until 17 December) | 0 | 91.1 c |

In 2013–14, we repeated the experiments conducted in 2012–13 and examined the effects of chilling amounts and the timing of the chilling exposure on flowering and bud breaking of the lateral flower buds. In all of the treatments, the flowering rate increased with longer chilling durations (Figure 5a). Significantly more chilling was required for endodormancy release as the starting time for chilling became earlier, and the flowering rate reached >70% when chilling accumulated for 2100, 1500, 1200 and 900 h, corresponding to the initiation of chilling on 7 October, 1 November, 4 December and 31 January, respectively. The starting time and amount of chilling similarly affected floret number per flower bud, and floret numbers increased as the trees were exposed to longer and later chilling temperatures (Figure 5b). In contrast, the bud breaking rates of lateral flower buds were much less affected when the chilling was initiated, and >70% bud breaking occurred when chilling accumulated for longer than 900 h irrespective of the timing of the chilling treatment (Figure 5a). These results, again, suggested that chilling provided earlier (November–December) might have less of an impact on endodormancy release to flowering.

In order to compare bud development during forcing more closely, we measured during forcing the outer length and flower cluster height of the lateral flower buds of trees chilled starting on 1 November for 1200 and 1500 h and of trees that were chilled starting on 4 December for 1200 h. Among these treatments, endodormancy was not released for the buds of trees chilled from 1 November for 1200 h (flowering <70%), but

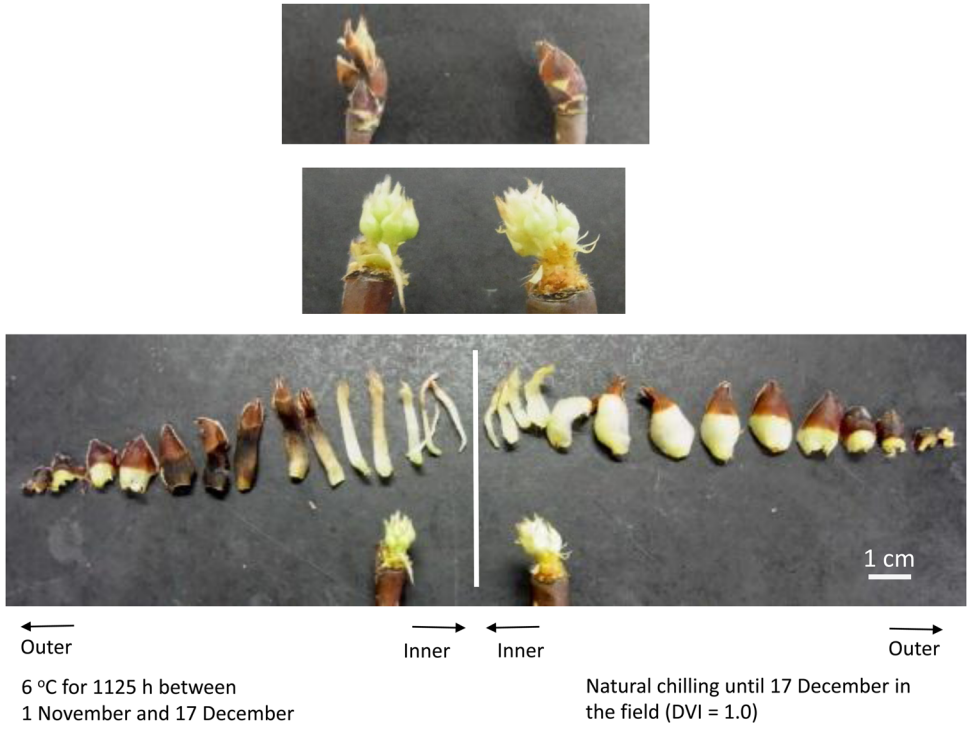


Figure 4. Intact (upper panel) and dissected (middle and lower panels) flower buds exposed to chilling in the dark between 1 November and 17 December (6 °C for 1125 h; left), and those that accumulated chilling under natural conditions until 17 December (DVI = 1.0 which correspond to 6 °C for 750 h; right) in 2012. These treatments are illustrated in Figure 2 (indicated with star). Photo was taken after 31 days of forcing at 15 °C.

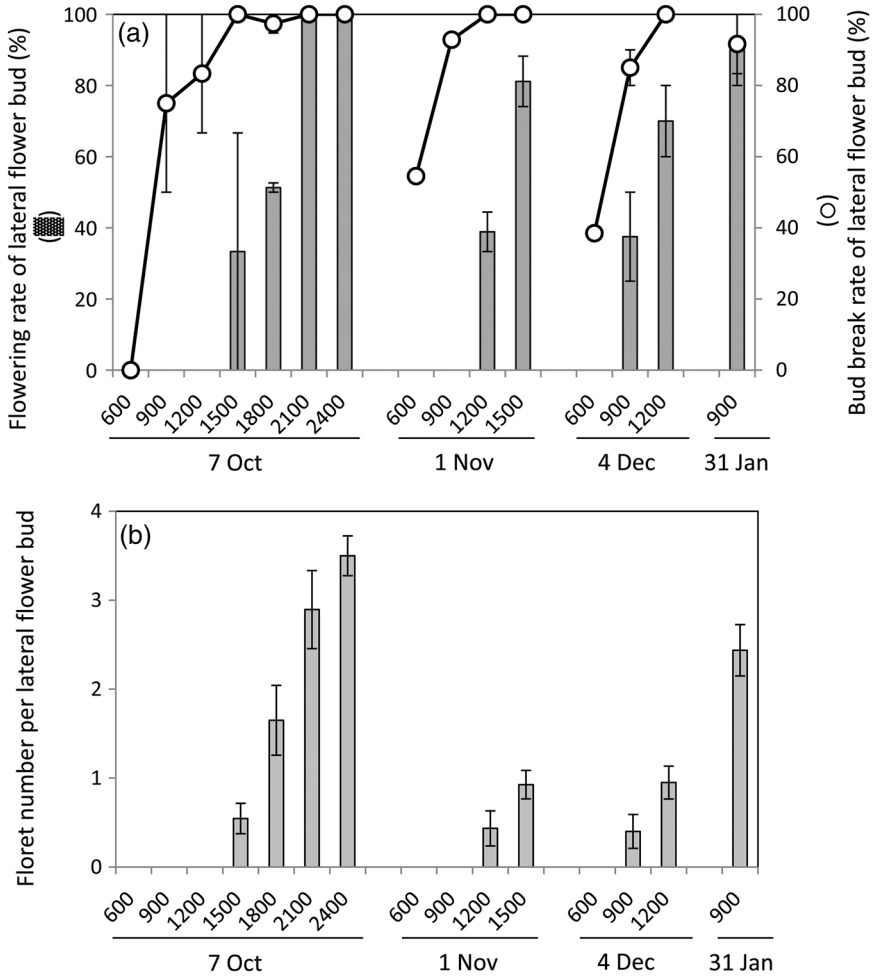


Figure 5. Effects of the amount and starting time of chilling on the flowering rate (bar graph, left vertical axis) and bud breaking rate (line graph, right vertical axis) of the lateral flower buds (a), and on the floret number per lateral flower bud (b) of potted trees of Japanese pear 'Kosui' in 2013–14.

endodormancy was released for buds from the other two treatments (Figure 5). Outer bud length was much more elongated in the trees chilled for 1500 h than those for 1200 h irrespective of when chilling was initiated (Figure 6), suggesting that scale leaf elongation during forcing depends on the number of chilling hours. On the other hand, flower cluster height increased in the following order: trees chilled in November for 1200 h, trees chilled in November for 1500 h and trees chilled in December for 1200 h. This result suggests that both the number of chilling hours and the timing of the chilling influence floret growth and development during forcing. Neither outer bud length nor flower cluster height increased in the trees chilled for 600 h irrespective of the timing.

### *Expression of dormancy- and flowering-related genes*

During chilling accumulation in 2012–13, the expression of pear *DAMs* (*PpMADS13-1*, *13-2* and *13-3*) in lateral flower buds decreased as chilling accumulated, irrespective of when chilling was imposed (Figure 7). In addition, *PpTFL1-1a* in lateral buds that commenced chilling on 1 November was expressed at higher levels as the duration of chilling increased, but expression was not affected by the other starting times for chilling initiation regardless of the amount of chilling. On the other hand, chilling did not affect the expression of *PpFT1a*, *PpFT2a* and *PpTFL1-2a* during chilling; however, the expression of *PpFT2a* and *PpTFL1-2a* 'before' exposure to chilling (i.e., buds at 0 h of chilling) was higher when chilling was disturbed for a longer period and peaked in January and February, respectively.

The expression of pear *DAMs* (*PpMADS13-1*, *13-2* and *13-3*) in lateral flower buds decreased as chilling accumulated, irrespective of the timing of chilling in both 2012–13 (Figure 7) and 2013–14 (Figure 8). In addition, *PpTFL1-1a* in the lateral buds was expressed at higher levels as the duration of chilling increased, and the degree of increase became larger as the time interval for chilling was earlier. *PpFT2a* expression in the lateral buds increased when trees accumulated chilling for longer than 2100 h with the chill beginning on 7 October. There were no consistent differences in *PpFT1a* and *PpTFL1-2a* expression among the three treatments.

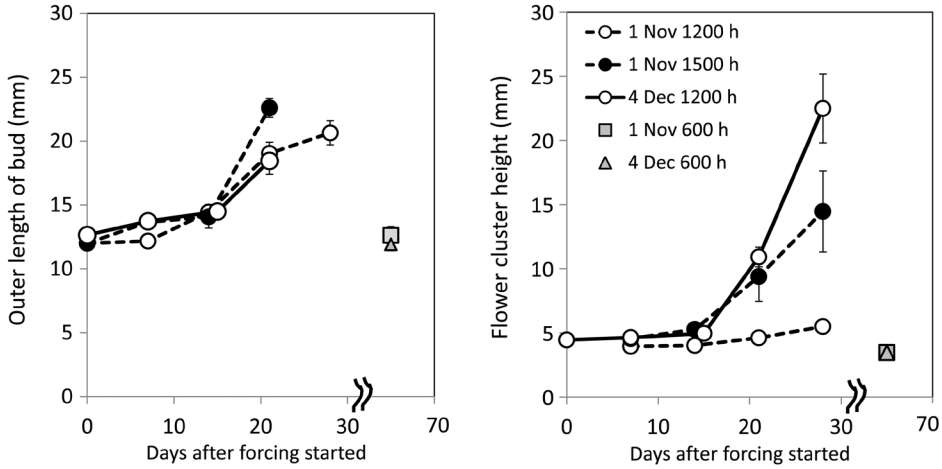


Figure 6. Comparison of the outer length (left) and flower cluster height (right) of the lateral flower buds chilled starting on 1 November for 1200 h, chilled starting on 1 November for 1500 h and chilled starting on 4 December for 1500 h during the 28 days of the forcing treatment (flowering rate of these treatments was 39, 81 and 70%, respectively, and is shown in Figure 7). For comparison, the data from buds chilled starting on either 1 November or 4 December for 600 h are also shown. Outer length of the buds chilled from 1 November for 1500 h and that from 4 December for 1200 h after 28 days of forcing was not measured because the scale leaves had already fallen and the florets had started elongating ($n = 10$, mean ± SE).

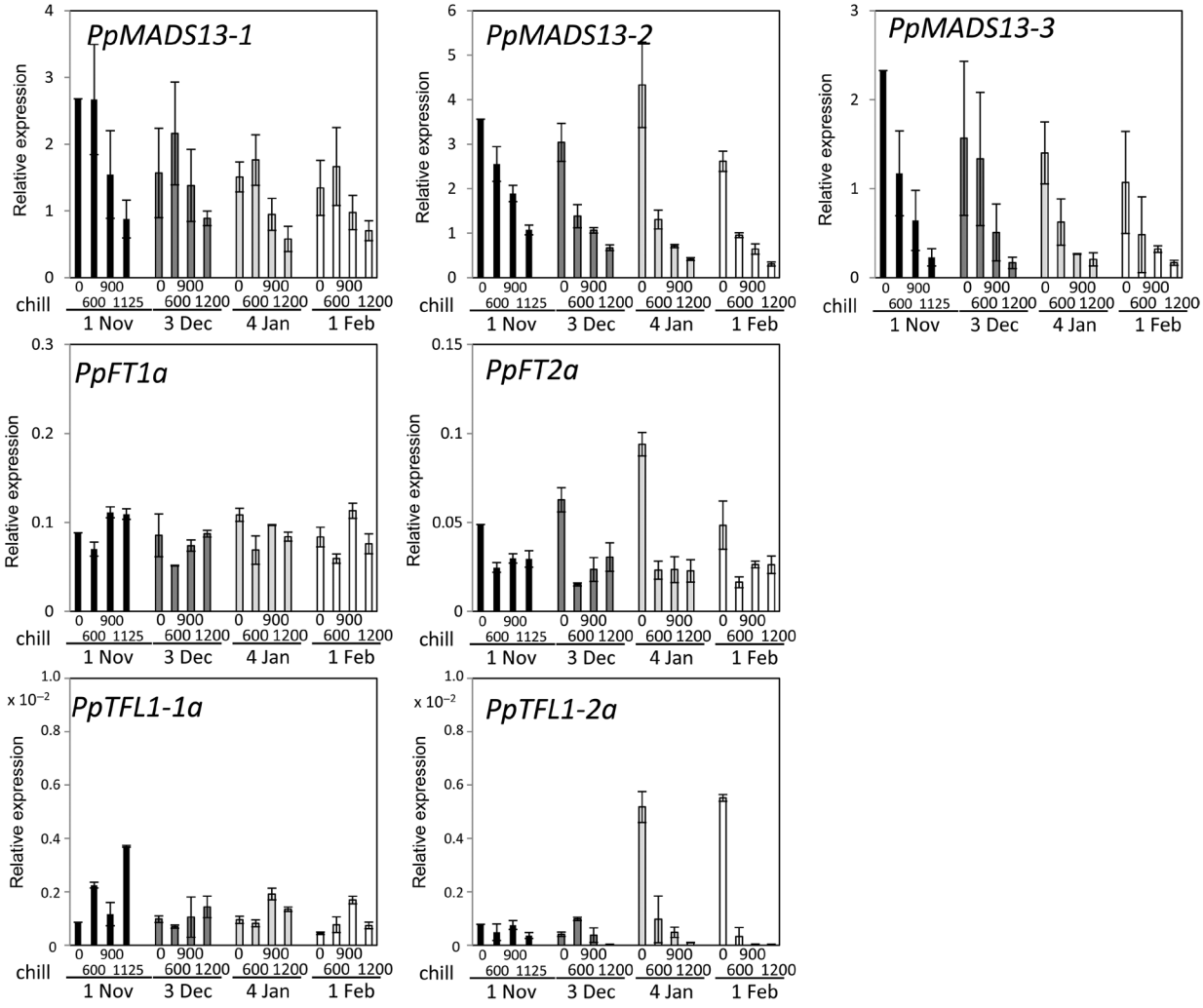


Figure 7. Effects of the amount and starting time of chilling on the expression of flowering- and dormancy-related genes during chilling accumulation in the lateral flower buds of Japanese pear 'Kosui' in 2012–13. Gene expression was measured by reverse transcription qPCR using the *HistoneH3* gene as a reference ($n = 2$, mean ± measurement range).

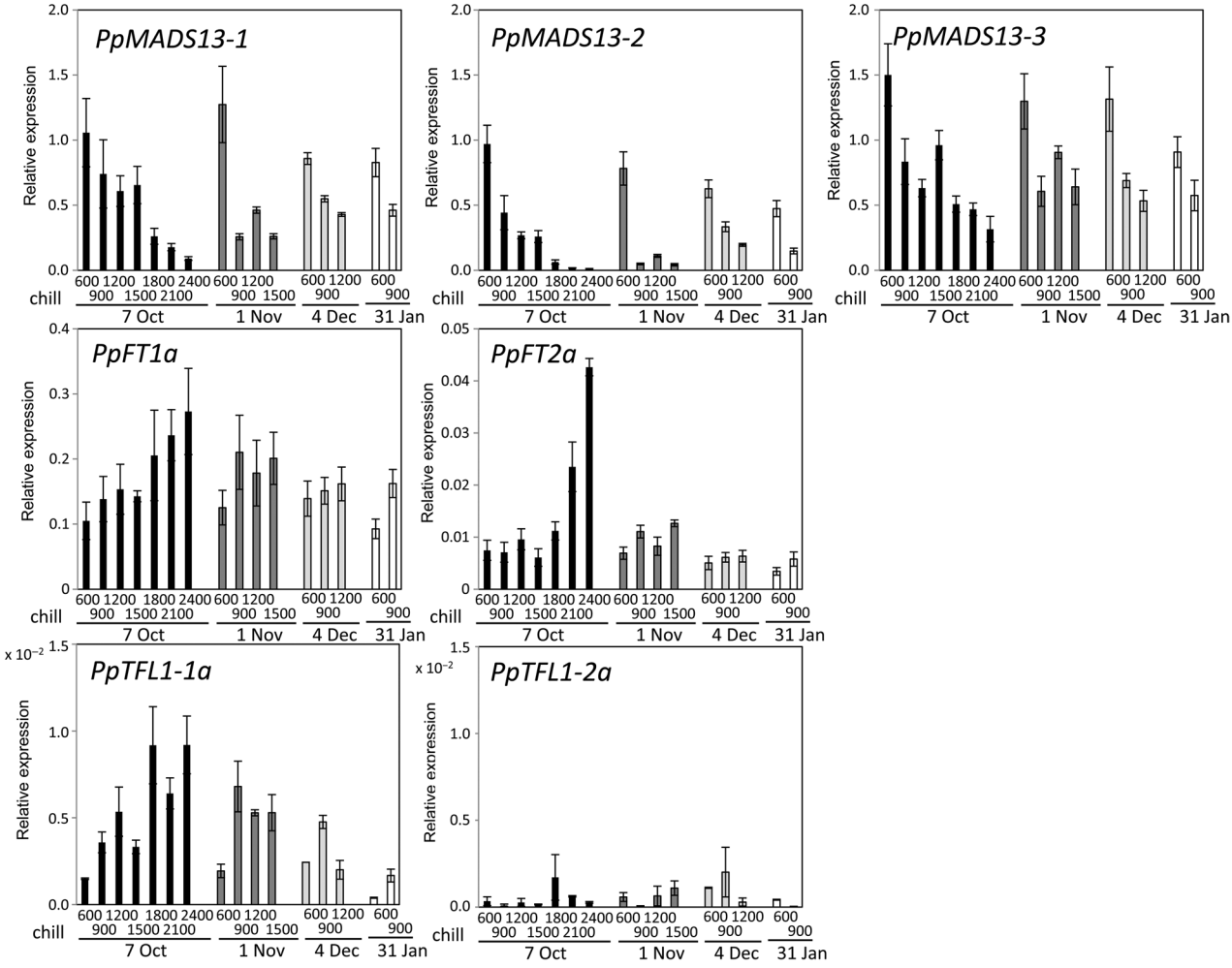


Figure 8. Effects of the amount and starting time of chilling on the expression of flowering- and dormancy-related genes during chilling accumulation in the lateral flower buds of Japanese pear 'Kosui' in 2013–14. Gene expression was measured by reverse transcription qPCR using the *HistoneH3* gene as a reference ($n = 2$, mean ± measurement range).

On the other hand, during the forcing treatment, the expression of *PpMADS13-1* and *13-3* in lateral flower buds decreased following transfer of the trees to 15 °C (Figure 9). Buds of trees chilled on 1 November for 1500 h had lower levels of expression of all three isogenes than those chilled beginning on 1 November and 4 December for 1200 h. In addition, expression of *PpFT2a* in the buds increased as the duration for which the trees were forced became longer, but the order of increasing expression was for trees chilled starting on 1 November for 1500 h, trees chilled starting on 4 December for 1200 h and trees chilled starting on 1 November for 1200 h. This result corresponded positively with flower cluster height during forcing (Figure 6). There were no consistent differences in *PpTFL1-1a* and *PpTFL1-2a* expressions among these three treatments.

## Discussion

In perennial trees, endodormancy is released when the trees are exposed to a sufficient amount of chilling that is genetically determined (e.g., Fan et al. 2010, Saito et al. 2013). However, our experiment clearly showed that chilling had different effects on bud breaking and flowering in Japanese pear lateral flower buds. Since chilling was provided under completely dark conditions in our experiments, it is possible that trees might respond differently to chilling under a natural photoperiod and light intensity. Chilling to ~900 h at 6 °C in the dark always induced bud breaking (scale elongation) when provided between October and February, whereas chilling provided earlier (between October and December) was less effective on flowering (floret growth and development) than later chilling. Similarly, different effects of chilling provided in different seasons on the fulfillment of chilling in trees was reported previously (e.g., reviewed in Luedeling 2012, and literature cited therein). In addition, the existence of qualitatively different phases of chilling in different periods of dormancy progression is also suggested in hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.); 3 weeks of chilling induces 'canonical bud burst', i.e., leaf protrusion and unfolding, but 4 weeks of chilling is capable of inducing 'true bud burst',

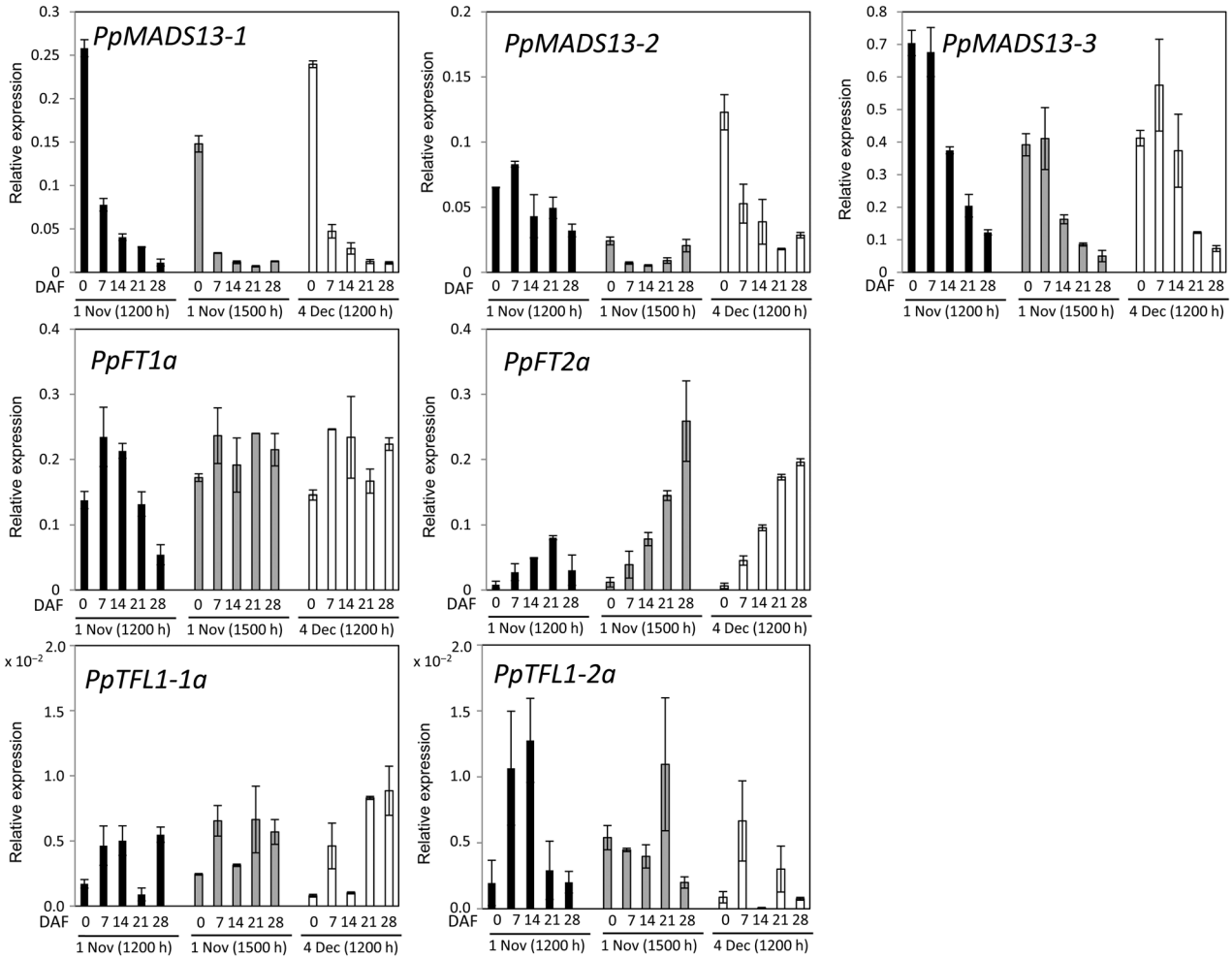


Figure 9. Effects of the amount and starting time of chilling accumulation on the expression of flowering- and dormancy-related genes during the forcing treatment in the lateral flower buds of temperature-treated Japanese pear 'Kosui' in 2013–14 (DAF: days after forcing). Gene expression was measured by reverse transcription qPCR using the *HistoneH3* gene as a reference ($n = 2$, mean ± measurement range).

which is accompanied by the formation of leaves and stem elongation (Rinne et al. 2011). In hybrid aspen, chilling induces *FT* expression and reopens signal conduits that allow the FT protein to access the meristematic apex. Subsequently, these two processes coordinate to release dormancy.

A close relationship between the expression of the *DAM* genes and the endodormancy phase transition has been reported. Expression of the *DAM* genes increases toward deep endodormancy, decreases concomitant with endodormancy release (Li et al. 2009, Ubi et al. 2010, Yamane et al. 2011) and is regulated via histone modification in their promoter region (Ruttink et al. 2007, Leida et al. 2012, Saito et al. 2013, 2015). In Japanese apricot (*Prunus mume* Sieb. et Zucc.), ectopic expression of a *DAM* gene (*PmDAM6*) in transgenic poplar (*P. tremula* L. × *P. tremuloides* Michx.) inhibits apical growth during the actively growing season (Sasaki et al. 2011), implying that *PmDAM6* in Japanese apricot may participate in the inhibition of bud growth during dormancy though it might not be the single determinant of bud dormancy (Yamane 2014). Notably, several investigators have used leaf buds, but not flower buds, of Rosaceae plants to show the correlation between endodormancy progression and *DAM* gene expression (Li et al. 2009, Ubi et al. 2010, Yamane et al. 2011). In our 2-year replicated experiment, the expression of *PpMADS13-1*, *13-2* and *13-3* decreased with longer exposures to chilling irrespective of the timing (Figures 7 and 8). These expression patterns seemed in accordance with the bud breaking rate rather than with flowering. In addition, during the forcing treatment, buds chilled starting on 1 November for 1500 h expressed the lowest level of the three *PpMADS13* isogenes and had the most elongated scale leaves in comparison with buds chilled starting on either 1 November or 4 December for 1200 h. This result also suggests the possible involvement of *DAM* genes in the inhibition of bud breaking (scale leaf elongation). Further investigations of the precise roles of these *DAM* genes are required.

In winter annuals or biennial herbaceous plants like Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) and wheat (*Triticum aestivum* L.), floral transition competency demands a long-term (over

4–8 weeks) cold exposure, a phenomenon known as 'vernalization' (Chouard 1960). In *Arabidopsis*, vernalization is mediated by a transcription factor *FLOWERING LOCUS C* (*FLC*) that acts as a repressor of flowering (Michaels and Amasino 1999, Michaels et al. 2005, Pajoro et al. 2014) and whose expression is downregulated in response to prolonged cold (Sheldon et al. 2000, Aikawa et al. 2010). Recently, microarray analysis in apple discovered two transcription factors, *FLC*-like and *MADS AFFECTING FLOWERING* (*MAF*), whose expression levels are highly related to dormancy release (Porto et al. 2015). Interestingly, the expression of an apple *FLC*-like gene was induced toward dormancy release, in contrast to the *Arabidopsis FLC* that is highly expressed during winter. Thus, different roles for apple *FLC*-like and *Arabidopsis FLC* are suggested. So far, a true functional ortholog of *FLC* has not been reported in trees (Leseberg et al. 2006) including members of the Rosaceae family (Mouhu et al. 2009, Guitton et al. 2012). Instead, in *Populus*, the *CO*/*FT* module, a well-known component playing a critical role in flowering induction, is reported to play an important role in the transition from the active to the endodormant phase (Böhlenius et al. 2006, Hsu et al. 2011, Rinne et al. 2011). Rohde et al. (2011) identified six robust quantitative trait loci (QTLs) for the timing of bud set that were conserved in four different pedigrees, and *FT* colocalized with one of these QTLs. In our experiments, higher levels of expression of *PpFT2a* during the forcing (Figure 9) were found in buds with taller flower clusters (Figure 6), which suggests the positive involvement of *PpFT2a* in the regulation of chilling fulfillment for flowering. Similarly, *MdFT2*, the most homologous gene to *PpFT2a* in apple, is expressed highly in advance of flowering and might be related to the development of apple floral organs (Kotoda et al. 2010). On the other hand, during chilling, *PpTFL1-1a* was expressed at a higher level in the buds of trees chilled earlier and required longer chilling for flowering (Figures 7 and 8). *TFL1* is involved in repressing flowering (Bradley et al. 1996, Ratcliffe et al. 1998, 1999, Boss et al. 2004), probably by competing with FT for FD binding (Hanano and Goto 2011). In the regulation of flowering in the Rosaceae, *TFL1* is a major repressor that regulates seasonal flowering within the yearly growth cycle (Iwata et al. 2012, Koskela et al. 2012). Furthermore, the seasonal regulation of the *TFL1* mRNA level is a key mechanism for timely floral development (Mimida et al. 2011, Ito et al. 2014). Mohamed et al. (2010) reported that overexpression of *TFL1* increased the chilling requirements and delayed bud breaking of field-grown poplar. *TFL1*(s) in Japanese pear may also be similarly involved in increasing the chilling requirement, and the higher expression levels of *TFL1s* in the early chilling treatment may force trees to require a longer chilling period for endodormancy release. Accordingly, we postulate that the increase in *FT* expression may be necessary to induce flowering after chilling, whereas *TFL1s* may impair the chilling efficiency, although how they interact in endodormancy regulation remains unclear. Further elucidation of the precise roles and interactions of *FTs* and *TFL1s* in the regulation of flowering season and properties is required in the future.

Plants are able to keep track of time and to integrate this information with the perception of external environmental cues; this internal time-keeping system is known as the circadian clock (Shin et al. 2013). The circadian clock may be integrated into the internal calendar in a seasonal context (circannual rhythm) and regulate annual events such as flower induction, growth cessation, leaf shed and dormancy (Visser et al. 2010, and literature cited therein). The circannual rhythm gates the signaling of stimuli and limits the timing of maximum responsiveness to a specific season of the year. Thus, when stimuli of the same strength are applied at different times of the season, responses of different intensities may result, which are one of the consequences of circannual control (Hotta et al. 2007, Visser et al. 2010). Our experiment clearly showed that chilling in October and November has less impact for flowering than those beginning in December and later (Table 1 and Figure 5). We infer that the chilling input may be gated circannually; increases in the expression of *PpFT2a* and *PpTFL1-2a* in lateral flower buds (0 h in Figure 7) by delaying chilling until January or February may be related to the seasonal differences in the plants' signal sensing/transducing pathways for flowering. Other investigations also support the possible involvement of circadian and/or circannual rhythms in endodormancy regulation of trees (e.g., in poplar (*P. tremula* L. × *P. alba* L.): Ruttink et al. 2007, Ibáñez et al. 2010, in chestnut (*Castanea sativa* Mill.): Ramos et al. 2005, Allona et al. 2008 and in pear: Nishitani et al. 2012). It will be of interest to investigate the impact of chilling on endodormancy progression of perennial plants in light of circannual rhythms. In addition, reexamination of the effect of chilling efficiency on flowering under a natural photoperiod may provide clearer insight into the circannual regulation of endodormancy progression in woody perennial plants.

## Conclusions

Flowering time of Japanese pear lateral flower buds is much more finely regulated than that for bud breaking. Flowering may be mediated via the balance of two flowering-related genes *FT* and *TFL1*, whereas bud breaking may be mediated via the *DAM* genes. We infer that chilling may be the primary cue for endodormancy release allowing the lateral flower bud to break; however, other supplementary cue(s) may allow the fine-tuning of flowering time. Our results suggest the possibility that the impact of chilling on flowering competence may be gated by a circannual rhythm through regulating *FT* and *TFL1* expression, although the mechanism by which plants sense the seasons remains unknown. Reconsidering endodormancy release and flowering competence in light of circannual rhythms may lead to new insights into the adaptation mechanisms of woody perennial plants.

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## Conflict of interest

None declared.

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## References

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

Allona I, Ramos A, Ibáñez C, Contreras A, Casado R, Aragoncillo C (2008) Molecular control of winter dormancy establishment in trees: a review. Span J Agric Res 6:201–210.

Anderson JV, Gesch RW, Jia Y, Chao WS, Horvath DP (2005) Seasonal shifts in dormancy status, carbohydrate metabolism, and related gene expression in crown buds of leafy spurge. Plant Cell Environ 28: 1567–1578.

Bielenberg DG, Wang Y, Li Z, Zhebentyayeva T, Fan S, Reighard GL, Scorza R, Abbott AG (2008) Sequencing and annotation of the evergrowing locus in peach [*Prunus persica* (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. Tree Genet Genomes 4:495–507.

Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O (2006) *CO/FT* regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 312:1040–1043.

Boss PK, Bastow RM, Mylne JS, Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell 16:S18–S31.

Bradley D, Vincent C, Carpenter R, Coen E (1996) Pathways for inflorescence and floral induction in *Antirrhinum*. Development 122: 1535–1544.

Chouard P (1960) Vernalization and its relations to dormancy. Annu Rev Plant Physiol 11:191–238.

Cooke JEK, Eriksson ME, Junttila O (2012) The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. Plant Cell Environ 35:1707–1728.

Fan S, Bielenberg DG, Zhebentyayeva TN, Reighard GL, Okie WR, Holland D, Abbott AG (2010) Mapping quantitative trait loci associated with chilling requirement, heat requirement and bloom date in peach (*Prunus persica*). New Phytol 185:917–930.

Guitton B, Kelner J-J, Velasco R, Gardiner SE, Chagnè D, Costes E (2012) Genetic control of biennial bearing in apple. J Exp Bot 63:131–149.

Hanano S, Goto K (2011) *Arabidopsis TERMINAL FLOWER1* is involved in the regulation of flowering time and inflorescence development through transcriptional repression. Plant Cell 23:3172–3184.

Heide OM, Prestrud AK (2005) Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol 25:109–114.

Horvath D (2009) Common mechanisms regulate flowering and dormancy. Plant Sci 177:523–531.

Hotta CT, Gardner MJ, Hubbard KE, Baek SJ, Dalchau N, Suhita D, Dodd AN, Webb AAR (2007) Modulation of environmental responses of plants by circadian clocks. Plant Cell Environ 30:333–349.

Hsu CY, Adams JP, Kim H et al. (2011) *FLOWERING LOCUS T* duplication coordinates reproductive and vegetative growth in perennial poplar. Proc Natl Acad Sci USA 108:10756–10761.

Ibáñez C, Kozarewa I, Johansson M, Ögren E, Rohde A, Eriksson ME (2010) Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in *Populus* trees. Plant Physiol 153:1823–1833.

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. doi:10.1371/journal.pone.0086492

Ito A, Saito T, Nishijima T, Moriguchi T (2014) Effect of extending the photoperiod with low-intensity red or far-red light on the timing of shoot elongation and flower-bud formation of 1-year-old Japanese pear (*Pyrus pyrifolia*). Tree Physiol 34:534–546.

Iwata H, Gaston A, Remay A et al. (2012) The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. Plant J 69:116–125.

Koskela EA, Mouhu K, Albani MC et al. (2012) Mutation in *TERMINAL FLOWER1* reverses the photoperiodic requirement for flowering in the wild strawberry *Fragaria vesca*. Plant Physiol 159:1043–1054.

Kotoda N, Hayashi H, Suzuki M et al. (2010) Molecular characterization of *FLOWERING LOCUS T*-like genes of apple (*Malus* × *domestica* Borkh.). Plant Cell Physiol 51:561–575.

Lang GA (1987) Dormancy—a new universal terminology. HortScience 22:817–820.

Leida C, Conesa A, Llácer G, Badenes ML, Ríos G (2012) Histone modifications and expression of DAM6 gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. New Phytol 193:67–80.

Leseberg CH, Li A, Kang H, Duvall M, Mao L (2006) Genome-wide analysis of the MADS-box gene family in *Populus trichocarpa*. Gene 378:84–94.

Li Z, Reighard GL, Abbott AG, Bielenberg DG (2009) Dormancy-associated MADS genes from the EVG locus of peach [*Prunus persica* (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. J Exp Bot 60:3521–3530.

Luedeling E (2012) Climate change impacts on winter chill for temperate fruit and nut production: a review. Sci Hortic 144:218–229.

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11:949–956.

Michaels SD, Himelblau E, Kim SY, Schomburg FM, Amasino RM (2005) Integration of flowering signals in winter-annual *Arabidopsis*. Plant Physiol 137:149–156.

Mimida N, Ureshino A, Tanaka N et al. (2011) Expression patterns of several floral genes during flower initiation in the apical buds of apple (*Malus* × *domestica* Borkh.) revealed by in situ hybridization. Plant Cell Rep 30:1485–1492.

Mohamed R, Wang C-T, Ma C et al. (2010) *Populus CEN/TFL1* regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*. Plant J 62:674–688.

Mouhu K, Hytönen T, Folta K, Rantanen M, Paulin L, Auvinen P, Elomaa P (2009) Identification of flowering genes in strawberry, a perennial SD plant. BMC Plant Biol 9:122. doi:10.1186/1471-2229-9-122

Nishitani C, Saito T, Ubi BE, Shimizu T, Itai A, Saito T, Yamamoto T, Moriguchi T (2012) Transcriptome analysis of *Pyrus pyrifolia* leaf buds during transition from endodormancy to ecodormancy. Sci Hortic 147:49–55.

Pajoro A, Biewers S, Dougali E et al. (2014) The (r)evolution of gene regulatory networks controlling *Arabidopsis* plant reproduction: a two-decade history. J Exp Bot 65:4731–4745.

Pérez FJ, Kühn N, Vergara R (2011) Expression analysis of phytochromes A, B and floral integrator genes during the entry and exit of grapevine-buds from endodormancy. J Plant Physiol 168:1659–1666.

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

Ramos A, Perez-Solis E, Ibañez C, Casado R, Collada C, Gómez L, Aragoncillo C, Allona I (2005) Winter disruption of the circadian clock in chestnut. Proc Natl Acad Sci USA 102:7037–7042.

Ratcliffe OJ, Amaya I, Vincent CA, Rothstein S, Carpenter R, Coen ES, Bradley DJ (1998) A common mechanism controls the life cycle and architecture of plants. Development 125:1609–1615.

Ratcliffe OJ, Bradley DJ, Coen ES (1999) Separation of shoot and floral identity in *Arabidopsis*. Development 126:1109–1120.

Rinne PLH, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjärvi J, van der Schoot C (2011) Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1,3-β-glucanases to reopen signal conduits and release dormancy in *Populus*. Plant Cell 23:130–146.

Rohde A, Storme V, Jorge V et al. (2011) Bud set in poplar—genetic dissection of a complex trait in natural and hybrid populations. New Phytol 189:106–121.

Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, Fromm J, Bhalerao RP, Boerjan W, Rohde A (2007) A molecular timetable for apical bud formation and dormancy induction in poplar. Plant Cell 19:2370–2390.

Saito T, Bai S, Ito A, Sakamoto D, Saito T, Ubi BE, Imai T, Moriguchi T (2013) Expression and genomic structure of the dormancy-associated MADS box genes *MADS13* in Japanese pears (*Pyrus pyrifolia* Nakai) that differ in their chilling requirement for endodormancy release. Tree Physiol 33:654–667.

Saito T, Bai S, Imai T, Ito A, Nakajima I, Moriguchi T (2015) Histone modification and signalling cascade of the dormancy-associated MADS-box gene, *PpMADS13-1*, in Japanese pear (*Pyrus pyrifolia*) during endodormancy. Plant Cell Environ 38:1157–1166.

Sasaki R, Yamane H, Ooka T, Jotatsu H, Kitamura Y, Akagi T, Tao R (2011) Functional and expressional analyses of *PmDAM* genes associated with endodormancy in Japanese apricot. Plant Physiol 157:485–497.

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES (2000) The molecular basis of vernalization: the central role of *FLOWERING LOCUS C (FLC)*. Proc Natl Acad Sci USA 97:3753–3758.

Shin J, Anwer MU, Davis SJ (2013) Phytochrome-interacting factors (PIFs) as bridges between environmental signals and the circadian clock: diurnal regulation of growth and development. Mol Plant 6:592–595.

Sugiura T, Honjo H (1997) The effects of temperature on endodormancy completion in Japanese pear (*Pyrus pyrifolia* Nakai) and modeling the relationship. J Agric Meteorol 53:285–290.

Takemura Y, Sudo S, Ikeda T, Matsumoto K, Tamura F (2011) Chilling induces bud endodormancy in Japanese pear 'Gold Nijisseiki'. Hortic Res (Jpn) 10:87–92.

Ubi BE, Sakamoto D, Ban Y et al. (2010) Molecular cloning of dormancy-associated MADS-box gene homologs and their characterization during seasonal endodormancy transitional phases of Japanese pear. J Am Soc Hortic Sci 135:174–182.

Visser ME, Caro SP, van Oers K, Schaper SV, Helm B (2010) Phenology, seasonal timing and circannual rhythms: towards a unified framework. Philos Trans R Soc B Biol Sci 365:3113–3127.

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 Jpn Soc Hortic Sci 83:187–202.

Yamane H, Tao R, Ooka T, Jotatsu H, Sasaki R, Yonemori K (2011) Comparative analyses of dormancy-associated MADS-box genes, PpDAM5 and PpDAM6, in low- and high-chill peaches (*Prunus persica* L.). J Jpn Soc Hortic Sci 80:276–283.