Expression Analysis of Endodormancy and Flowering-related Genes in Greenhouse-cultivated Flowering Disorder Trees of Japanese pear (Pyrus pyrifolia Nakai) ‘Kosui’

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The emergence of flowering disorder, specifically the bud break disorder observed in flower buds, has become a serious problem for Japanese pear ‘Kosui’ greenhouse production in southern Japan. To understand the mechanism behind this problem, the expression of genes related to endodormancy and flowering were investigated in “flowering disorder trees” (FDTs). In 2017 and 2019, remarkably warm temperatures were recorded during the winter season, and the degree of flowering disorder in FDTs was severe. Forced cultivation experiments suggested that endodormancy of “normal trees” (NTs) was released well before fulfilling the chilling requirement (CR), suggesting that ‘Kosui’ trees grown in greenhouses had a short endodormancy period. We also found that the dormancy depth of FDTs was shallower than that of NTs before and after fulfilling the CR, whereas FDTs entered a deeper dormancy when the greenhouse was covered by a plastic film during the ecodormancy period, suggesting that FDTs’ dormancy progression was significantly different from that of NTs during endodormancy and ecodormancy. Gene expression analysis showed that the expression level of the endodormancy-related gene, MADS13-3, was correlated with dormancy depth in NTs, but not in FDTs. Additionally, the expression levels of the putative flowering regulator, PpFLC3-like gene, were significantly lower in FDTs than in NTs in December, before fulfilling the CR. Additionally, the flowering promoter, PpFT, was also significantly lower in FDTs than in NTs in March after fulfilling the CR. The survey years 2017 and 2019 were warm winters with a high mean temperature difference from the climatological normal in December. These results suggest that the flowering disorder of greenhouse-cultivated ‘Kosui’ is related to the shallow dormancy depth and/or inappropriate dormancy and flowering progression during endodormancy and ecodormancy, especially when flower buds encounter high temperatures during dormancy.

Key Words: DAM, FLC, flower buds, high temperature, sleeping symptom.

Introduction

Japanese pear (Pyrus pyrifolia Nakai) is an important deciduous fruit tree cultivated from Kyushu to Hokkaido in Japan. ‘Kosui’, ‘Hosui’, and ‘Niitaka’ are the main varieties, with ‘Kosui’ having the largest cultivation area at 40.2% of all varieties (MAFF, 2017 <https://www.maff.go.jp/j/tokei/kouhyou/tokusan_kazuyu/>). ‘Kosui’ is shipped by taking advantage of climate differences and forced cultivation in each region from West to East Japan from July to September. Particularly, in Kyushu, ‘Kosui’ cultivation involves greenhouses, and August shipments secure high unit prices.

However, the emergence of flowering disorder, a bud break disorder of flower buds, has recently become a serious problem for ‘Kosui’ production, especially regarding greenhouse-cultivated Japanese pears in the Kyushu area (Tominaga et al., 2019). Flowering disorder is also known as “sleeping symptom” among farmers, because the trees appear to remain asleep even during the normal blooming season. The main symptoms of flowering disorder are as follows: (1) delayed blooming throughout the trees; (2) flower bud abortion; (3) only the tip of the axillary flower buds show bud break, but no bud break is observed in other lateral flower buds on the branches; (4) blooming of flowers with short and/or weak peduncles; (5) alternate floret abortion and vegetative shoot formation; and (6)
bloom of only a few, small florets. In greenhouse cultivation, numerous severe symptoms occur throughout the trees (Tominaga et al., 2019).

Flowering disorder in the greenhouse-cultivated ‘Kosui’ has been observed since the early 2000s (Fujimaru, 2004; Matsuda, 2004). Although there are annual differences in the degree of occurrence, flowering disorder is typically identified in greenhouse cultivation every year. In Fukuoka Prefecture, greenhouse-cultivated ‘Kosui’ accounts for approximately 25% of the total ‘Kosui’ cultivation area (Fukuoka Prefecture, 2016 <https://www.pref.fukuoka.lg.jp/gyosei-shiryo/tokusankaju30.html>). The presence of flowering disorder is strongly affected by unseasonably high temperatures in autumn and winter, plastic film covering timing, and variations in greenhouse temperature after plastic film covering (Tominaga et al., 2019). On another note, in open field-cultivated Japanese pear, flowering disorder occurred in various areas of Japan in 2009 (NARO, 2015 <https://www.naro.affrc.go.jp/org/niaes/ccaf/project2015/manual2015/nihon-nashi_2015.pdf>). However, after that almost no flowering disorder occurred until it was identified again primarily in Kagoshima Prefecture in southern Japan in 2016 (Ito et al., 2018), which was primarily due to the instability of endodormancy induction affected by unseasonably high temperatures in autumn and winter (Ito et al., 2018), as well as the decreased freezing tolerance of dormant branches due to nitrogen application in autumn and winter (Sakamoto et al., 2017). Flowering disorder of greenhouse-cultivated trees may also be affected by unstable endodormancy because flowering disorder symptoms were similar between greenhouse- and open field-cultivated ‘Kosui’ (Tominaga et al., 2019). However, to date, no detailed physiologic or molecular studies have been conducted to understand flowering disorder of greenhouse-cultivated ‘Kosui’ trees.

Endodormancy is a physiologic mechanism that prevents growth in winter and can be released when appropriate chilling temperatures are accumulated (Lang, 1987). This is called the chilling requirement (CR), and buds can shift from endodormancy to ecodormancy when the CR is achieved. Then, buds can shift from ecodormancy to the bud breaking period after exposure to subsequent warm conditions. For Japanese pears, during normal flowering approximately eight florets bloom from one compound flower bud accompanied by leaf sprouting. When bud break occurs before exiting endodormancy, flowering becomes delayed or irregular (Saure, 1985). Recently, the molecular mechanisms of endodormancy and flowering in Rosaceae fruit trees have been well studied. In Japanese pear, the expression of dormancy-related MADS-box (DAM) genes increased with induction and decreased with the breaking of endodormancy (Saito et al., 2013, 2015; Yang et al., 2020). It was reported that the increased expression of the Flowering locus T (FT) gene induces flowering after breaking endodormancy (Ito et al., 2016). Furthermore, in the apple species (Malus × domestica Borkh.) that is closely related to Japanese pear, the dormancy-related (Daccord et al., 2017; Kumar et al., 2016; Nishiyama et al., 2019; Porto et al., 2015) Flowering Locus C (FLC) genes are proposed to primarily control flowering progression (Kagaya et al., 2020; Zong et al., 2019). However, regarding flowering disorder of greenhouse-cultivated Japanese pears, there is currently no knowledge of the molecular mechanisms of endodormancy and flowering.

In the present study, we analyzed the expression of endodormancy- and flowering-related genes of flowering disorder trees in greenhouse-cultivated ‘Kosui’ to gather fundamental knowledge in order to develop flowering disorder control technology. First, we made efforts to find “flowering disorder trees” (FDTs) that had experienced severe symptoms for three consecutive years at a greenhouse in Asakura City, Fukuoka Prefecture. Then, we used these trees to analyze the flowering rate, dormancy depth before and after CR fulfillment, and dormancy- and flowering-related gene expression of FDTs for two years.

Materials and Methods

Outline of survey greenhouse
From November 2016 to February 2017 and November 2018 to March 2019, Japanese ‘Kosui’ pears were surveyed at greenhouses A and B in Asakura City, Fukuoka Prefecture. Greenhouse A corresponds to the greenhouse orchard termed “B” in our previous study, and greenhouse B corresponds to half of the greenhouse orchard termed “A” in that study (Tominaga et al., 2019), in which numerous cases of flowering disorder are confirmed every year. All surveyed trees were over 35 years old. An outline of the cultivation conditions is provided in Figure 1. A heater equipped with ducts was used to heat the greenhouse to approximately 5°C overnight, and automatic ventilation was provided via ventilating fans and intake ports throughout the day. Cultivation conditions were based on the standards of the JA Chikuzen Asakura Pear committee, although the management programs of greenhouses A and B differed depending on the farmer.

Selection of “Flowering Disorder Trees”
Trees with lower flowering rates were evaluated as more severe in terms of flowering disorder occurrence. In the present study, trees with a consecutive flowering rate of 60% or less from 2014 to 2016 were defined as FDTs, and trees with a flowering rate of over 70% were defined as “normal trees” (NTs) and were used in subsequent tests (Fig. 2A, B). The numbers of trees used for the investigation were 159 and 76 in greenhouses A and B, respectively (Fig. 2A, B).

The flowering rate, that is, the proportion of flowering buds to the total buds per tree at full bloom (when
Fig. 2. FDTs selection in 2014–2016. Numbers of trees with less than 60% flowering rate in (A) greenhouse A and (B) greenhouse B. n indicates the total number of trees investigated in the greenhouse. The black bar graph shows the number of FDTs with a flowering rate of less than 60% for three consecutive years.

Fig. 1. Cultivation flow of the surveyed greenhouses.

Flowering rate of “Flowering Disorder Trees” in the greenhouses

During the flowering period in March 2017, the flowering rates of three FDTs (Tree ID, #A1, #A2, #A3) and three NTs (Tree ID, #A4, #A5, #A6) in greenhouse A were investigated. Additionally, during the flowering period in March 2019, the flowering rates of one FDT (Tree ID, #B1) and one NT (Tree ID, #B2) in greenhouse B were investigated.

Dormancy depths of “Flowering Disorder Trees”

To investigate dormancy depth, a temporal flowering test of cut branches was conducted as previously described by Fadón et al. (2020) and Liu et al. (2012), with slight modifications. In the present study, the bud break rate of lateral flower buds was observed and used as a dormancy depth indicator. From November 2016 to February 2017, two shoots (50–100 cm long) were sampled from three FDTs (#A1, #A2, #A3) and three NTs (#A4, #A5, #A6) in greenhouse A. For shoots before defoliation in November, the attached leaves were artificially removed. Two consecutive axillary flower buds were collected from the shoots and placed in rock wool that was submerged in distilled water. The shoots were incubated at 25°C under 16 h of light. After three weeks, the flowering rate was investigated and indexed for dormancy depth as follows: Blooming at 0, bud break just before blooming at −1, immediately after bud break at −2, and absence of bud break at −3 (Fig. 5A). The average value of two branches was taken to predict the dormancy depth for each tree. We considered that the lower the value, the deeper the dormancy. Then, the average value of three trees was used as the dormancy depth for NTs and FDTs.

Expression patterns of endodormancy- and flowering-related genes in “Flowering Disorder Trees”

From November 2016 to February 2017, the lateral flower buds of long fruit branches were sampled seven times from three FDTs (#A1, #A2, #A3) and three NTs (#A4, #A5, #A6) in greenhouse A. From November 2018 to March 2019, the lateral flower buds of long fruit branches were sampled seven times from one FDT (#B1) and one NT (#B2) in greenhouse B. The lateral flower buds were stored at −30°C or −80°C.

Quantitative gene expression analysis was conducted on the axillary flower bud samples. DAM genes ([PpMADS13-1] (accession number: AB504716), [PpMADS13-2] (AB504717), and [PpMADS13-3] (AB774474.1)) were analyzed as endodormancy-related genes. Additionally, one FLC gene, a homolog of MdFLC3-like in apple that is a closely related species to Japanese pear, was analyzed. MdFLC3 and MdFLC3-like act as a flowering repressor and promoter, respectively (Kagaya et al., 2020; Zong et al., 2019). We found a Japanese pear FLC homologous gene (AB524589) registered in the NCBI database and defined it as a [PpFLC3-like] gene because it showed the highest similarity to MdFLC3 (Kagaya et al., 2020) and MdFLC3-like (Zong et al., 2019) genes. We analyzed [PpFLC3-like] as a candidate flowering regulator gene, although the detailed function of the gene is unknown. The [PpFT2a] gene (AB571595) was also analyzed as a flowering promoter gene because FT is a well-known flowering promoter in many plant species (Kobayashi et al., 1999). [HistoneH3] (AB824718) was used as an in-

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ternal standard gene. The specific primers used were those previously reported by Saito et al. (2013) for *PpMADS13-1*, *PpMADS13-2*, and *PpMADS13-3* and those reported by Ito et al. (2014) for *PpFT2a*. For *PpFLC3*-like, specific primers were prepared at the Universal ProbeLibrary Assay Design Center (Roche Life Science) based on the CDS sequence, and primer sets of 5'-TCATAGAAATGAGATTCAGAC-3' and 5'-TTCCTAGTCGGCAAGTGTGTCAG-3' were used.

Total RNA was extracted using the phenol–chloroform method using RNAiso Plus (Takara Bio) after freezing the axillary flower buds with liquid nitrogen, followed by grinding. The obtained total RNA was then subjected to DNase treatment using DNase I (RNase-Free; New England Biolabs). Then, cDNA was synthesized from 100 ng of total RNA using a PrimeScript RT Regent Kit (Perfect Real Time; Takara Bio). Quantitative gene expression analysis was conducted on 10 μL of the reaction mixture containing 1 × SYBR Premix Ex Taq III, 0.8 μM of forward primer, 0.8 μM of reverse primer, and 2 μL of cDNA. Additionally, only the *PpFLC3*-like gene was used in a 10 μL reaction solution containing 1 × SYBR Premix Ex Taq III, 0.06 μM of forward primer, 0.06 μM of reverse primer, and 2 μL of cDNA. The PCR reaction and data collection were conducted using a Thermal Cycler Dice Real Time System II (Takara Bio) with 40 cycles of 95°C for 5 s and 60°C for 30 s after heat denaturation at 90°C for 30 s. After one cycle of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s, the melting curve was analyzed to confirm whether the data obtained were derived from the target genes. Finally, agarose gel electrophoresis was conducted to confirm whether the PCR product was derived from the target gene.

**Correlation between dormancy depth- and endodormancy-related gene expression**

Correlation analysis was conducted on dormancy depth and the expression levels of endodormancy-related genes in NTs and FDTs before plastic film covering. From November 2016 to February 2017, the dormancy depth and the expression levels of endodormancy-related genes were measured seven times, and each datum was plotted on a scatter plot corresponding to the survey date. The correlation coefficients R and P were calculated using Pearson’s product moment correlation analysis.

**General weather conditions**

Using the AMeDAS data (Asakura) of the Japan Meteorological Agency, we extracted the mean temperature difference from the climatological normal and the daily maximum temperature for the aforementioned survey years. Additionally, a developmental index (DVI) model was used to predict the CR fulfillment day (Sugiura and Honjo, 1997). The average hourly temperature obtained from the AMeDAS data in the survey area from November was integrated, and the day on which DVI = 1.0 was set as the CR fulfillment date.

**Results**

**Selection of “Flowering Disorder Trees”**

To select FDTs, the proportion of trees with a low flowering rate (60% or less) were investigated in greenhouses A and B. In greenhouse A, the numbers of trees with low flowering rate were 71, 100, and 35 trees in 2014, 2015, and 2016, respectively. We classified 23 trees that showed low flowering rates for three consecutive years as FDTs (Fig. 2A). The ratio of FDTs to the number of trees with a low flowering rate in the year with the lowest proportion (2016; 35 trees) was 65.7%. In greenhouse B, the numbers of trees with low flowering rate were 39, 14, and 40 trees in 2014, 2015, and 2016, respectively. We assigned 12 trees as FDTs (Fig. 2B). The ratio of FDTs to the number of trees with a low flowering rate in the year with the lowest proportion (2015; 14 trees) was 85.7%. From FDTs, three trees (#A1, #A2, #A3) in greenhouse A and one tree (#B1) in greenhouse B were selected for the present study. From NTs with a flowering rate of more than 70% for three consecutive years, three trees (#A4, #A5, #A6) in greenhouse A and one tree (#B2) in greenhouse B were also selected for the study.

**Flowering rate of “Flowering Disorder Trees” in the greenhouses**

In 2017, for greenhouse A the three NTs displayed flowering rates of 80% to 90%, whereas the three FDTs showed flowering rates of 20% to 30% (Fig. 3A). In 2019, for greenhouse B, one NT showed a flowering rate of 90%, whereas one FDT showed a flowering rate of 50% (Fig. 3B). Images of NTs and FDTs showing 90% and 20% flowering rates, respectively, are shown in Figure 4A and 4B.

Compared with the NT flowers (Fig. 4C), flowering disorder in FDT was observed by delayed flower bud break (Fig. 4D), dwarf flower organs (Fig. 4E), a decreased number of florets (Fig. 4F), flower bud abortion
Changes in dormancy- and flowering-related gene expression during 2016-2017 in greenhouse A. Relative expression of (A) PpMADS13-1, (B) PpMADS13-2, (C) PpMADS13-3, (D) PpFLC3-like, and (E) PpFT2a genes. Relative expression was normalized to PpHistoneH3 expression level. The vertical bar indicates standard error (n = 3–4 axillary flower bud repetitions). Different letters indicate significant differences at a 5% significance level using the Tukey–Kramer test. Graphs without letters indicate no significant difference using the Tukey–Kramer test.

**Dormancy depths of “Flowering Disorder Trees”**

The endodormancy depth of FDTs in greenhouse A showed a slight decrease on December 2; however, it remained at more than −0.5 from November 2016 to February 2017 before plastic film covering (Fig. 5B). The endodormancy depth of NTs decreased to −2.5 on November 11 and increased on December 22, and decreased again on February 3, reflecting ecodormancy induction, then increased once more after plastic film covering, suggesting that ecodormancy was released due to plastic film covering (Fig. 5B). The endodormancy and ecodormancy depths before plastic film covering were always lower for FDTs than for NTs (Fig. 5B).

**Expression patterns of endodormancy- and flowering-related genes in “Flowering Disorder Trees”**

The expressions of endodormancy-related genes (PpMADS13-1, PpMADS13-2, and PpMADS13-3) and flowering-related genes (PpFLC3-like and PpFT2a) were measured. PpMADS13-1 and PpMADS13-2 gene expression levels in NTs and FDTs in greenhouse A were high from November 11 to December 2 from 2016 to 2017; however, there was no significant temporal change (Fig. 6A, B). In NTs, the expression levels of PpMADS13-3 peaked on November 11, showing significantly different temporal changes. Thereafter, expression levels decreased once on December 2 and slightly increased from January 13 to February 3 (Fig. 6C). On another note, PpMADS13-3 expression in FDTs showed a temporal change similar to that in NTs; however, no
significant difference was observed in the expression levels between FDTs and NTs (Fig. 6C). *PpFLC3-like* expression levels in NTs peaked on December 2 and showed significantly different temporal changes. Thereafter, expression levels sharply decreased once on December 22 and then slightly increased on January 13 (Fig. 6D). *PpFLC3-like* expression levels in FDTs were highest on December 2 and were significantly lower than those in NTs (Fig. 6D). Thereafter, the expression levels of the *PpFLC3-like* gene in FDTs decreased from December 22. Furthermore, *PpFT2a* gene expression levels peaked on February 27 in FDTs and were significantly lower than those in NTs (Fig. 6E).

In greenhouse B from 2018 to 2019, the expression levels of the *PpMADS13-1* and *PpMADS13-2* genes in NT and FDT were highest on December 25; however, there was no significant temporal change (Fig. 7A, B). Additionally, *PpMADS13-3* gene expression levels peaked on December 25 in NT and showed significant temporal changes and then decreased after January 9 (Fig. 7C). The expression levels of *PpMADS13-3* in FDT were higher on December 25, as in the NT. However, there was no significant difference between FDT and NT in terms of *PpMADS13-3* expression (Fig. 7C). The expression levels of the *PpFLC3-like* gene peaked on December 25 in NT and showed significant temporal changes and then decreased after January 9 (Fig. 7D). *PpFLC3-like* expression levels in FDT were highest on December 25 and were significantly lower than those in NT (Fig. 7D). Furthermore, the expression levels of the *PpFT2a* gene peaked on March 1 in NT and were significantly higher than those in FDT (Fig. 7E).

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**Fig. 7.** Changes in dormancy and flowering-related gene expression during 2018-2019 in greenhouse B. Relative expression of (A) *PpMADS13-1*, (B) *PpMADS13-2*, (C) *PpMADS13-3*, (D) *PpFLC3-like*, and (E) *PpFT2a* genes. Relative expression was normalized to *PpHistone3* expression level. The vertical bar indicates standard error (n = 3 axillary flower bud repetitions). Different letters indicate significant differences at a 5% significance level using the Tukey–Kramer test. Graphs without letters indicate no significant difference via the Tukey–Kramer test.

**Fig. 8.** Correlation between dormancy depth and the relative expression of (A) *PpMADS13-3* gene in NTs and (B) *PpMADS13-3* gene in FDTs in greenhouse A. Dormancy depth and gene expression levels of NTs and FDTs from 2016 to 2017 are plotted. R: correlation coefficient by Pearson’s product moment correlation analysis.

**Correlation between dormancy depth and gene expression**

The correlation between dormancy depth and endodormancy-related gene expression from 2016 to 2017 was analyzed. The correlation coefficient between the expression levels of *PpMADS13-3* and the dormancy depth of NTs was −0.6963, indicating a significant negative correlation (Fig. 8A). However, the correlation coefficient was 0.0715 between *PpMADS13-3* expression levels and dormancy depth in FDTs (Fig. 8B).

**General weather conditions**

The sum of the mean temperature difference from climatological normal temperature between the survey years was +90.1°C from November 2016 to February 2017 (Fig. 9A) and +134.7°C from November 2018 to February 2019 (Fig. 9B), indicating a warm winter trend. The maximum temperature on the day with the maximum mean temperature difference was 20.0°C on
The mean temperature difference from the climatological normal (A, B) and maximum temperatures (C, D) during 2016–2017 (A, C) and 2018–2019 (B, D).

In the present study, we analyzed endodormancy- and flowering-related gene expression by focusing on greenhouse-cultivated FDTs to obtain fundamental knowledge regarding flowering disorder mechanisms.

In greenhouses A and B, where flowering disorder occurs yearly according to a previous report (Tominaga et al., 2019), there were 23 FDTs trees (trees with flowering disorder for three consecutive years) in greenhouse A and 12 trees in greenhouse B (Fig. 2A, B). The ratios of FDTs to the number of trees with a low flowering rate in the year with the lowest proportion were 65.7% in greenhouse A (2016) and 85.7% in greenhouse B (2015). The higher ratio of FDTs suggests that although the number of trees with flowering disorder varied depending on annual environmental changes, flowering disorder may tend to recur in the same trees in greenhouses.

The flowering rate of FDTs was less than 50% in both the 2016–2017 and 2018–2019 growing seasons, and flowering was significantly delayed compared with NTs (Fig. 4). Additionally, the expression of the flowering-related gene PpFT2a was significantly lower in FDTs immediately before blooming (February 27, 2017, and March 1, 2019) than in NTs (Figs. 6E and 7E). As PpFT2a has the highest homology with MdFT2 in apples and is predicted to mediate reproductive organ development (Kotoda et al., 2010), it was determined that organ development in the flower buds was delayed in FDTs.

In the dormancy depth survey, endodormancy became deeper in NTs on November 11, 2016, and NTs presumably entered endodormancy at this time. Thereafter, the endodormancy depth decreased on December 22, suggesting that endodormancy was released at this time. As December 22 was earlier than the expected CR fulfillment date, that is, January 7, as predicted by the DVI model, it was suggested that endodormancy was short in NTs (Fig. 5B). Although it is still unclear how and why endodormancy was shortened in the tested trees, the environmental and/or cultural conditions related to a greenhouse forced cultivation system may have affected the endodormancy progression of the tested trees. In FDTs, although the endodormancy was slightly deeper on December 2, it remained at a lower level until February 3, and a clear endodormancy period could not be determined. After CR fulfillment, the dormancy depth of NTs increased again, which may reflect ecodormancy induction in January and February, whereas FDTs did not enter ecodormancy in that period. FDTs entered deep dormancy after the trees were covered with plastic film. Thus, we concluded that dormancy in FDTs was shallower than in NTs during the endodormancy and ecodormancy periods. However, FDTs entered dormancy in an unusual period, that is, the end of February, when endodormancy and ecodormancy were released in NTs. These results suggested that the dormancy progression of FDTs was significantly different from that of NTs.

It was found that PpMADS13 expression was in-
creased during endodormancy, and its expression decreased toward the break of endodormancy (Saito et al., 2013, 2015; Yang et al., 2020). MADS13-3 expression levels were highest in NTs on November 11; however, they decreased significantly from December 2, before increasing again from January 13 to February 3 (Fig. 6C). The seasonal changes in endodormancy and ecodormancy depths showed a negative correlation with the expression levels of the endodormancy-related gene, PpMADS13-3, in NTs (Fig. 8). In previous studies, PpMADS13-1, PpMADS13-2, and PpMADS13-3 expression levels were all decreased toward the break of endodormancy from November to February (Saito et al., 2013), suggesting their significant roles during endodormancy. However, they found different expression patterns between PpMADS13-3 and PpMADS13-1, -2, suggesting that specific transcriptional control may be present in PpMADS13-3. In the present study, we found that only PpMADS13-3 showed a significant increase during endodormancy (Figs. 5 and 6), although all three PpMADS13 genes were relatively preferentially expressed during endodormancy. Additionally, our results suggested that PpMADS13-3 could be related not only to endodormancy depth, but also to ecodormancy depth in NTs. Furthermore, PpMADS13-3 expression levels were not correlated with dormancy depth in FDTs, which further suggested the abnormal dormancy progression of FDTs during endodormancy and ecodormancy.

It has been reported that DAM (PpMADS13) gene expression levels were decreased because of prolonged high-temperature treatment (25°C) (Saito et al., 2013). The days with the highest mean temperature difference to climatological normal temperatures were December 22, 2016, and December 4, 2018, with maximum temperatures of 20.0°C and 24.2°C, respectively (Fig. 9C, D). The flower bud sampling dates closest to these days were December 22, 2016, and December 11, 2018, and PpMADS13-3 gene expression levels on each day were very low in NTs (Figs. 6C and 7C). Thus, we hypothesized that the decreased expression levels of PpMADS13-3 in December were caused by exposure to temperatures over 20°C. Further studies will be required to understand the relationship between transient high-temperature exposure and endodormancy progression. High temperatures reset the CR levels needed to break dormancy (devernalization) in vegetables such as radishes and model plants, including Arabidopsis (Bouche et al., 2015; Purvis and Gregory, 1945). This concept has also been reported for fruit trees (Erez, 2000; Sugiura et al., 2003, 2007). Based on previous studies and the results of gene expression analysis in the present study, the depth of endodormancy of NTs and FDTs may become shallower due to the resetting of CR levels caused by exposure to high temperatures.

PpFLC3-like was expressed during the end of endodormancy in NTs (Fig. 6D). PpFLC3-like is a homologous gene of MdFLC3 (Kagaya et al., 2020) and MdFLC3-like genes in apples (Zong et al., 2019). MdFLC3 represses flowering in Arabidopsis (Kagaya et al., 2020), whereas MdFLC3-like promoted flowering when overexpressed in blueberries (Vaccinium spp.) (Zong et al., 2019). Thus, PpFLC3-like may regulate flowering in Japanese pear, although it is still unknown whether it promotes flowering or represses it. The lower expression levels of PpFLC3-like in FDTs than in NTs (Figs. 6D and 7D) suggest that flower development activity during endodormancy differs between FDTs and NTs. We thus hypothesized that flowering progression may differ between FDTs and NTs, which may also influence flowering disorder occurrence in FDTs. Nishiyama et al. (2019) recently reported that MdFLC-like belonged to the FLC clade similar to MdFLC3-like, which inhibits vegetative growth in Arabidopsis and presumably plays a role as a growth inhibitor and bud break repressor during the end of endodormancy and ecodormancy in apples. Therefore, if PpFLC3-like has functions similar to MdFLC-like, the lower expression of PpFLC3-like in FDTs than in NTs may be related to lower dormancy depth at the end of endodormancy. Our expression analysis of PpMADS13-3 and PpFLC3-like collectively suggested that endodormancy and flowering progression may be different between NTs and FDTs, although further functional studies will be required to understand the physiologic function of PpFLC3-like.

Ito et al. (2018) reported that the instability of endodormancy causes flowering disorder. Based on changes in dormancy depth and gene expression in the present study, the depth of endodormancy in FDTs was considered to be shallower than that in NTs (Figs. 5 and 6). The endodormancy period is considered to be an important phase for nutrient storage for the following season’s bud outgrowth. For example, starch accumulation continues during endodormancy, and starch is believed to be the energy source for the following season’s growth after its conversion to sugar during dormancy release in sweet cherries (Prunus avium) (Fadón et al., 2018). The high temperatures during autumn and winter may have affected the endodormancy depth of FDTs, making it shallower than that of NTs, and flowering disorder may have been caused by the poor induction of endodormancy in FDTs. Based on previous and current studies, we hypothesized the possible mechanisms of flowering disorder as follows: (1) FDTs encounter high temperatures, resulting in shallow endodormancy. (2) This leads to abnormal endodormancy progression in FDTs. (3) Consequently, farmers cover FDTs with a plastic film after inappropriate dormancy progression. (4) The exposure to high temperatures due to the plastic film covering may cause flowering and bud break disorder, probably because of a lack of adequate growth-promoting factors, such as starch accumulation.

In conclusion, our results suggest that flowering dis-
order in greenhouse-cultivated Japanese pear is caused by a shallow depth of endodormancy or a delay in flowering. Additionally, it was demonstrated that gene expression analysis of endodormancy-related genes was effective in measuring the depth of endodormancy and the progress of flowering during endodormancy. A previous study found that high temperatures in autumn may become a more common occurrence because of global warming (Sugiura et al., 2007). Therefore, the manifestation of flowering disorder in both greenhouse- and open field-cultivated trees is concerning. In the future, a detailed analysis of the mechanisms of endodormancy and flowering of Japanese pear trees is needed to establish countermeasures against flowering disorder.

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