RNA-sequencing Analysis Identifies Genes Associated with Chilling-mediated Endodormancy Release in Apple

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ABSTRACT. Endodormancy release and the fulfillment of the chilling requirement (CR) are critical physiological processes that enable uniform blooming in fruit tree species, including apple (*Malus ×domestica*). However, the molecular mechanisms underlying these traits have not been fully characterized. The objective of this study was to identify potential master regulators of endodormancy release and the CR in apple. We conducted RNA-Sequencing (RNA-seq) analyses and narrowed down the number of candidates among the differentially expressed genes (DEGs) based on the following two strict screening criteria: 1) the gene must be differentially expressed between endodormant and ecodormant buds under different environmental conditions and 2) the gene must exhibit chill unit (CU)–correlated expression. The results of our cluster analysis suggested that global expression patterns varied between field-grown buds and continuously chilled buds, even though they were exposed to similar amounts of chilling and were expected to have a similar dormancy status. Consequently, our strict selection strategy resulted in narrowing down the number of possible candidates and identified the DEGs strongly associated with the transition between dormancy stages. The genes included four transcription factor genes, *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*), *FLOWERING LOCUS C (FLC)-LIKE*, *APETALLA2 (AP2)/ETHYLENE-RESPONSIVE 113 (ERF113)*, and *MYC2*. Their expressions were upregulated during endodormancy release, and were correlated with the CU, suggesting that these transcription factors are closely associated with chilling-mediated endodormancy release in apple.

Perennial woody plants in temperate regions modulate their growth rhythm to adapt to seasonal environmental changes. For example, bud dormancy is an adaptive mechanism that enables buds to survive unfavorable conditions, such as low temperatures during winter. Bud dormancy can be fundamentally defined as the inability of the meristem to resume growth under favorable conditions (Rohde and Bhalerao, 2007). Lang (1987) and Lang et al. (1987) classified the physiological states of dormant buds into the following three types: para-, endo-, and ecodormancy. Paradormancy is also known as correlative inhibition and is induced by signals produced in other parts of a growing plant. Endodormancy refers to a suspension of growth due to internal factors within the meristem-containing tissue. Endodormancy establishment is controlled by environmental cues, such as low temperatures and a short photoperiod (Maurya and Bhalerao, 2017; Olsen, 2010). A previous study revealed that low temperatures are the only requirement for endodormancy establishment in apple (Heide and Prestrud, 2005). Endodormant buds are incapable of resuming growth until they are exposed to a specific chilling period (i.e., CR), which is genetically determined. After the CR has been

satisfied, buds are released from endodormancy and shifted to ecodormancy, in which growth is inhibited by external environmental factors, such as cold and drought conditions. Despite these dormancy classifications, it is difficult to qualitatively discriminate between phases because there is some overlap between each phase. Thus, we currently cannot precisely distinguish between endodormancy and ecodormancy in trees.

Bud dormancy release and CR fulfillment are agronomically important physiological steps that influence flowering time and the subsequent fruit production. Dormancy-related flowering disorders (e.g., nonuniform flowering and bud abortion) that inhibit fruit production have been observed in many species and are believed to have been caused, at least in part, by insufficient endodormancy release putatively due to global warming (Sugiura et al., 2007). Consequently, the genetic factors and the molecular network underlying endodormancy release should be elucidated. A thorough characterization of the molecular basis of endodormancy release may enable the artificial control of endodormancy through cultural practices. It may also be relevant for efficient breeding (e.g., marker-assisted selection).

The molecular regulation of endodormancy has recently been widely studied in many species, including poplar (*Populus* sp.), which is a model perennial woody plant (Cooke et al., 2012; Maurya and Bhalerao, 2017; Rinne et al., 2010; Ruttink et al., 2007). In poplar, the CONSTANS (CO)/FLOWERING LOCUS T (FT) module, which is critical for flowering in arabidopsis

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(*Arabidopsis thaliana*), is involved in short photoperiod-induced growth cessation and bud set (Böhlenius et al., 2006). Hsu et al. (2011) reported that there are two *FT* genes with distinct roles affecting development. Rohde et al. (2011) identified six robust quantitative trait loci (QTLs) for time to bud set in four different pedigrees, and *FT* colocalized with one of these QTLs. In addition, *FT* expression is hyperinduced during chilling-induced dormancy release in poplar (Rinne et al., 2011), suggesting that *FT* is involved in both dormancy establishment and release. In addition, *FT* reportedly affects dormancy in grapevine (*Vitis* sp.) (Pérez et al., 2011). Other genes potentially affecting dormancy are *DORMANCY-ASSOCIATED MADS-BOX (DAM)* genes, which belong to the *SHORT VEGETATIVE PHASE (SVP)/AGAMOUS-LIKE 24 (AGL24)* clade. The *DAM* genes were first identified as candidate genes in the *evergrowing (evg)* peach (*Prunus persica*) mutant incapable of entering dormancy (Bielenberg et al., 2008). The *evg* mutant continues to grow even under dormancy-inducing conditions and exhibits a loss of transcriptional activity of six tandemly arrayed *DAM* genes (*DAM1–6*). The peach *DAM* gene expression levels were observed to be upregulated during endodormancy induction and downregulated during endodormancy release toward budbreak (Li et al., 2009; Yamane et al., 2011a, 2011b), suggesting these genes may influence dormancy progression and budbreak. In addition, a strong QTL for the CR and blooming date in peach was detected in the region near the *EVG* locus (Fan et al., 2010). Moreover, the possible involvement of *SVP*-like genes in either endodormancy induction or release was also reported for other perennial species, such as Japanese apricot [*Prunus mume* (Kitamura et al., 2016; Sasaki et al., 2011; Yamane et al., 2008)], Japanese pear [*Pyrus pyrifolia* (Ito et al., 2016; Saito et al., 2013; Ubi et al., 2010)], kiwifruit [*Actinidia* sp. (Wu et al., 2012)], raspberry [*Rubus idaeus* (Mazzitelli et al., 2007)], and leafy spurge [*Euphorbia esula* (Horvath et al., 2008)]. In apple, several *DAM*-like genes have been cloned, although different research groups have used different names for the same genes (Mimida et al., 2015; Porto et al., 2016; Wisniewski et al., 2015). Porto et al. (2016) proposed that the ‘Golden Delicious’ apple genome contains four *DAM*-like genes (*MdDAM1–4*). Expression analyses suggest that the *MdDAM* genes undergo seasonal expression-level changes, with an upregulation during endodormancy (Kumar et al., 2016a; Mimida et al., 2015; Porto et al., 2016; Wisniewski et al., 2015). However, in apple, the downregulated expression of *MdDAM* genes does not appear to be associated with the CR for endodormancy release (Mimida et al., 2015; Porto et al., 2016; Yamane et al., 2018), which is in contrast to *Prunus* species in which the downregulated expression levels of some *DAM* genes are associated with the genotype-dependent CR (Leida et al., 2012; Li et al., 2009; Sasaki et al., 2011; Yamane et al., 2011a, 2011b). The expression level of *MdDAM1*, which is the most highly expressed among all *MdDAM* genes, is relatively high even during endodormancy release but is subsequently downregulated during the budbreak stage (Mimida et al., 2015; Porto et al., 2016; Yamane et al., 2018). A recent finding that the ectopic expression of *MdDAMB*, which is one of the *SVP/DAM*-like genes other than *MdDAM1–4*, delays budbreak (Wu et al., 2017) suggests that *DAM* genes help regulate budbreak in both apple and *Prunus* species. However, previously reported endogenous expression analyses suggest that apple *DAM* genes may not be critical for endodormancy release.

Apple is one of the most important fruits in Japan and globally. Apple trees have adapted to survive in the relatively

cool climates of temperate regions. In Japan, apple flower buds differentiate in early summer and enter endodormancy in autumn, which is followed by a gradual shift to ecodormancy in midwinter. The effects of global warming may change where apple can be cultivated (i.e., more northern regions) and decrease apple production because of a dormancy-related disorder, which has already been observed in Brazil (Petri and Leite, 2004) and South Africa (Labuschagné et al., 2002). One characteristic of apple dormancy is that endodormancy is induced in response to low temperatures, but not to a short photoperiod (Heide and Prestrud, 2005), suggesting that the associated regulatory mechanism differs from that of other species that enter dormancy in response to a short photoperiod, such as poplar and peach. To date, apple dormancy has been studied from various perspectives, such as physiological and molecular changes (Porto et al., 2015; Wang et al., 1991; Wisniewski et al., 2015) as well as genetic analyses (Celton et al., 2011; van Dyk et al., 2010). All of these transcriptome studies involved the heterozygous ‘Golden Delicious’ apple genome (Velasco et al., 2010). However, the genome sequence of a homozygous GDDH13 doubled-haploid apple line was recently published (Daccord et al., 2017). The estimated number of apple genes has decreased from 63,541 in the original apple genome (Velasco et al., 2010) to 42,140 in the new apple reference genome (Daccord et al., 2017), and many overlapping genes have been filtered out. A transcriptome analysis using the new apple reference genome may enable more accurate mapping, ultimately resulting in a more precise identification of DEGs and gene ontology (GO) analysis. However, RNA-seq studies focused on apple dormancy have not been conducted using the new version of the apple genome.

The objective of the present study was to identify candidate master regulators responsible for controlling endodormancy release, such as *DAM* genes in *Prunus* species. So far, published transcriptome analyses of apple dormancy involving microarrays (Porto et al., 2015) or RNA-Seq (Kumar et al., 2016a) detected many DEGs as potential regulators of dormancy. In this study, we aimed to identify genes strongly associated with endodormancy release by narrowing down the number of candidates using a unique strategy. We applied the following strategy: 1) we sequenced mRNAs derived from samples exposed to several environmental conditions and collected an appropriate number of biological replications for each sampling date to enable statistical analyses; 2) we set two strict screening criteria: 1) the gene should be differentially expressed in the buds between endodormancy (0% budbreak under forcing conditions) and ecodormancy (100% budbreak under forcing conditions) and 2) the expression of the gene should be correlated with the CR for endodormancy release. We first conducted a cluster analysis to observe global gene expression-level changes in dormant buds under different environmental conditions. We then narrowed down the possible DEGs and identified a few transcription factors, candidates for potential master regulators of dormancy release in apple.

Materials and Methods

PLANT MATERIAL. This study was completed using six mature ‘Fuji’ apple trees grown at the experimental farm of the Department of Agriculture, Shinshu University (Nagano, Japan; 36° N and 139° E). The trees were grafted on either ‘JM1’ or ‘JM2’ rootstocks (both *Malus prunifolia*). The effects of each

rootstock on dormancy were not considered because our preliminary analysis indicated that their dormancy characteristics, such as bud burst dates, were almost the same (data not shown). The apple trees were grown using conventional cultural practices. In this study, we collected bud samples from the branches of trees grown under field conditions (2012–13 and 2014–15 seasons) or from excised branches exposed to an artificial chilling treatment. One-year-old middle branches (15–40 cm) with terminal flower buds were collected and immediately transferred to our laboratory at Kyoto University (Kyoto, Japan) after being wrapped in moistened paper and maintained at 4–10 °C. The basal parts of branches were then soaked in water containing 1% (v/v) cut flower preservation reagent (Misakifarm; Otsuka Kagaku, Tokushima, Japan), which contains nutrients and fungicides that can extend the life of a cut flower. The water was changed every week, and the basal ends of the branches were cut. We examined the terminal flower buds rather than the lateral or leaf buds because flower buds are more closely associated with fruit production than leaf buds and the timing and depth of lateral bud dormancy may differ depending on bud position (Faust et al., 1995; Saure, 1985), which prevents accurate predictions of dormancy status. Flower buds were distinguished from vegetative buds based on the fact they are larger.

CALCULATION OF THE CU. The accumulated CU under field and chilling conditions was calculated on each sampling date according to the Utah model (Richardson et al., 1974). The ambient temperature (hourly mean temperature) in the field was obtained from the nearest weather station (Japan Meteorological Agency, Ina, Japan). The chilling treatment involved maintaining the temperature at 5 °C (1 CU = 1 h at 5 °C).

INVESTIGATION OF THE SEASONAL CHANGES OF DORMANCY STATUS IN THE FIELD. In the 2012–13 season, branches were collected on the following dates: 16 Oct., 20 Nov., and 19 Dec. 2012, and 16 Jan. and 12 Mar. 2013. The accumulated CU for each month was 0, 434, 810, 994, and 1295, respectively. During the 2014–15 season, branches were collected on 8 Nov. 2014 and 9 Jan. 2015, with an accumulated CU of 170 and 825, respectively. The branches were artificially defoliated if they still contained leaves. They were then placed in growth chambers and incubated for 6 weeks at 22 °C under a 16/8-h (light/dark) photoperiod. The terminal flower bud burst rates were then recorded for at least five branches (i.e., biological replications). The bud burst date was defined as the date when green leaf tips first became visible. Two or three terminal flower buds (i.e., biological replications) excised from the branches on each sampling date were used to construct RNA-Seq libraries. The excised flower buds were immediately frozen in liquid nitrogen and stored at –80 °C until used.

INVESTIGATION OF THE DORMANCY STATUS OF BRANCHES UNDER CHILLING CONDITIONS. Branches were collected on 8 Nov. 2014 and artificially defoliated. They were subsequently incubated at 5 °C in darkness for 0, 10, 20, 30, 40, and 65 d. The branches were then transferred to growth chambers set at 22 °C under a 16-h light/8-h dark photoperiod. The accumulated CU for the 0, 10, 20, 30, 40, and 65 d of chilling treatment was 170, 410, 650, 890, 1130, and 1730, respectively. The terminal flower bud burst rates for four or five branches (i.e., biological replications) were recorded after 6 weeks in the growth chamber. Two or three branches (i.e., biological replications) that were chilled for 0, 10, 25, 35, and 65 d were used to construct RNA-Seq libraries. Terminal flower buds were collected and stored as described previously.

RNA EXTRACTION, LIBRARY CONSTRUCTION, AND RNA-SEQ.

Total RNA was extracted from the field-grown tree samples collected during the 2012–13 season using CTAB as described by Yamane et al. (2008). The extracted total RNA was sent to BGI Japan (Kobe, Japan) for the construction of the mRNA library and the subsequent sequencing (HiSeq. 2000 system, PE100; Illumina, San Diego, CA). The raw reads were filtered to remove adapter sequences, contaminants, and low-quality reads, and the sequences were split based on the barcode information.

Total RNA was extracted from the field-grown tree samples collected during the 2014–15 season and from the samples exposed to the artificial chilling treatment with the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA) and then purified with a phenol/chloroform extraction. An mRNA library was constructed as described by Akagi et al. (2014), with minor modifications. Briefly, mRNA was isolated from total RNA using the Dynabeads mRNA purification kit (Thermo Fisher Scientific, Waltham, MA). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and random primers. Second-strand cDNA was synthesized using the second-strand buffer (200 mM Tris-HCl, pH 7.0, 22 mM MgCl₂, and 425 mM KCl), DNA polymerase I (NEB, Ipswich, MA), and RNaseH (NEB). Double-stranded cDNA was purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Brea, CA), and then fragmented using NEBNext dsDNA Fragmentase (NEB). The fragmented DNA was subjected to end-repair, poly(A) tailing, adapter sequence ligation, size selection, and polymerase chain reaction enrichment. The resulting libraries were quantitatively and qualitatively assessed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit fluorometer (Invitrogen). The libraries were then sequenced using the Illumina HiSeq 2000 system (PE100). After filtering, to remove low-quality reads and contaminants, the resulting clean reads were divided based on the barcode information, after which the adapter sequences were eliminated.

CALCULATION OF THE READS PER KILOBASE PER MILLIONS (RPKM). Clean reads were mapped to the apple consensus mRNA sequences (Daccord et al., 2017) using the Burrows–Wheeler Aligner (Li and Durbin, 2009) with default parameters. The number of reads mapped to each gene was calculated using the Sequence Alignment/Map tool with custom R scripts. The raw read counts were normalized and converted to RPKM value.

CLUSTERING AND GO ENRICHMENT ANALYSIS. Before conducting the clustering analysis, the mean RPKM values of the biological replications were calculated for each condition, and genes with a low RPKM value or a low coefficient of variance (cv) score (mean RPKM < 1 or cv < 1 for all conditions) were removed. The remaining genes were hierarchically clustered using the Morpheus software with one minus the Pearson correlation metric and the average-linkage method, based on a Z-score matrix calculated according to the mean RPKM value for each condition.

Published functional annotations of the GDDH13 v1.1 genome mapped with the InterProScan database (Daccord et al., 2017) were retrieved and used in this study. A GO enrichment analysis was conducted using the goseq package v1.22.0 (Young et al., 2010) with default parameters. A false discovery rate (FDR) < 0.1 was used as the threshold for identifying significantly enriched GO terms, and GO terms with fewer than five genes in a given cluster were not employed in this study.

SCREENING OF DEGs TO IDENTIFY GENES CLOSELY ASSOCIATED WITH ENDODORMANCY RELEASE AND THE FULFILLMENT OF THE CR. We assumed that genes encoding putative master regulators of endodormancy release were differentially expressed between endo- and ecodormant buds. We also assumed that changes to the expression levels of endodormancy- and CR-related genes are associated with chilling accumulation because the shift from endo- to ecodormancy is theoretically correlated with chilling accumulation (Lang, 1987). The genes differentially expressed between endo- and ecodormant flower buds were analyzed using the DESeq R package (Anders and Huber, 2010). The FDR (Benjamini and Hochberg, 1995) and a threshold of 0.05 was used to identify DEGs. In addition, the Pearson correlation (r) between the gene expression value (RPKM) and the CU was calculated using Excel (Microsoft, Redmond, WA). Genes whose expression was statistically correlated with the CU ($|r| > 0.6$) were identified. The outline of our strategy is presented in Fig. 1. Genes selected according to our hypothesis were annotated based on a BLAST search of the nonredundant NCBI database. The genes with the highest matches were identified.

PHYLOGENETIC ANALYSIS OF THE APPLE FLC-LIKE GENE. A putative MADS-box-like gene was included among the screened genes. The FLC clade MADS-box genes were retrieved from genome sequences of apple, arabidopsis, peach, grape (*Vitis vinifera*), and poplar (*Populus trichocarpa*) using FLC from arabidopsis (Pařenicov et al., 2003) as query by BLAST search. Then, phylogenetic tree was constructed using the amino acid sequences of these retrieved genes and arabidopsis MADS-box genes (Pařenicov et al., 2003) according to the neighbor-joining method with 1000 bootstrap replications. The trees were prepared using the MEGA7 program (Kumar et al., 2016b), and were based on a sequence alignment using MAFFT version 7.0 (Katoh and Standley, 2013) and arrangement with trimAL (Capella-Gutirrez et al., 2009).

Results

DORMANCY STATUS OF BRANCHES UNDER CHILLING CONDITIONS. The results of our field analysis were consistent with the observations of a previous study, which revealed that ‘Fuji’ apple trees require about 1000 CU for endodormancy release (Hauagge and Cummins, 1991). Samples, Jan. 2013, Mar. 2013, and Jan. 2015 accumulated ≈ 1000 CU and had bud burst rates of 100% (Table 1; Fig. 2A and B). By contrast, under artificial chilling conditions, the highest bud burst rate was 60% after 40 d, even though the samples accumulated about 1000 CU (Fig. 2C). To identify the genes differentially expressed between the endodormancy and ecodormancy stages, samples were classified as endodormant, ecodormant, or in transition (i.e., from endodormancy to ecodormancy) based on the recorded bud burst rate under forcing conditions. Bud burst rates of 0% and 100% were defined as endo- and ecodormancy, respectively. For the RNA-Seq analysis, four sampling time points [Oct. 2012, Nov. 2012, Nov. 2014 (0 d), and 10 d] represented endodormancy, whereas three sampling time points (Jan. 2013, Mar. 2013, and Jan. 2015) corresponded to ecodormancy.

RNA-SEQ, MAPPING TO A NEW APPLE REFERENCE GENOME, AND CLUSTER ANALYSIS. Eleven samples were analyzed, with each consisting of two or three biological replications. About 0.5 billion clean reads (100 nucleotides long) were obtained, with

each sample comprising 27–68 million reads (Table 1). With the exception of one sample with an extremely low mapping rate (28.2% for 0 d-C), the average mapping rate was 78.2%, reflecting sequence differences between the ‘GDDH13’ (used to construct the reference genome) and ‘Fuji’ (used in this study) genomes (data not shown). The data for 0 d-C were excluded from subsequent analyses. All the obtained sequences from Illumina sequencing reads are available from the NCBI/ DDBJ Sequence Read Archive under the following accession numbers, DRA006592 (PRJDB6779).

A cluster analysis divided the expressed genes into eight clusters based on the global expression patterns (Fig. 3). The genes in cluster 1 were highly expressed at 10 and 25 d after chilling but their expression levels were downregulated at 35 and 65 d. By contrast, the expression levels of the cluster 2 genes were upregulated in response to a continuous chilling condition. The cluster 1 and 2 genes did not exhibit differential expression under a natural chilling condition. However, the expression levels of the genes in clusters 3–8 did not significantly change under a continuous chilling condition but underwent global changes under a natural chilling condition. The expression levels of genes in clusters 3 and 8 were considerably upregulated in samples collected in Jan. 2015 and 2013, respectively. Meanwhile, cluster 4 gene expression levels were up-regulated in Mar. 2013, whereas the expression levels of genes in clusters 5 and 6 were up-regulated in Nov. 2014 and 2012, respectively. The expression levels of genes in clusters 4–6 were generally down-regulated in samples collected in December and January. By contrast, the cluster 7 gene expression levels were upregulated in October and December but downregulated from January onward.

The enriched GO terms in each cluster are listed in Table 2. No GO terms were significantly enriched in clusters 3, 5, 6, and 8. However, in cluster 4, GO terms related to cell division, such as “DNA replication” and “regulation of cell cycle,” were

Fig. 1. Outline of the strategy used to identify potential regulators of apple endodormancy release. Samples were treated with natural or artificial chilling conditions. Candidate genes were narrowed down according to the following criteria: 1) the gene should be differentially expressed in the buds between endodormancy (0% budbreak under forcing conditions) and ecodormancy (100% budbreak under forcing conditions) and 2) the expression of the gene should be correlated with the chilling accumulation required for endodormancy release.

enriched, suggesting that active bud outgrowth started in March. The GO terms related to oxidative stress were enriched in clusters 2 and 7, which consisted of genes whose expression levels were up-regulated in response to a prolonged cold treatment under continuous and natural chilling conditions, respectively. This suggested that responses to oxidative stress, including enhanced peroxidase activity, may contribute to the

physiological changes induced by a prolonged exposure to low temperatures, such as dormancy release, cold adaptation and hardening or both.

SCREENING OF DEGs. Our analyses with 12 pairwise comparisons ($FDR < 0.05$), using four endo- and three ecodormancy samples revealed that 184 genes were differentially expressed in common in all comparisons (data not shown), even though 4039–20,488 DEGs were identified in each comparison (Table 3). The Pearson correlation (r) between the RPKM value and the CU was calculated for $\approx 44,953$ mapped genes in the apple genome. We detected ≈ 5522 genes whose expression was highly correlated with the CU ($|r| > 0.6$). More than 95% of the correlations were positive, with negative correlations observed for only 237 genes. Of the 184 genes that were differentially expressed, the expression levels of 13 and 31 were positively and negatively correlated with the CU, respectively (Table 4). These genes were annotated based on

Table 1. Details regarding the samples from field-grown trees and chilling treatment used for our apple dormant buds RNA-Seq analyses.

Sample name	Chill units	Replications (no.)	Total quality reads (no.)	Total mapped reads (no.)
Oct. 2012	0	2	45,375,330	42,390,109
Nov. 2012	434	3	56,812,376	50,305,934
Dec. 2012	810	2	43,860,040	40,353,212
Jan. 2013	994	2	68,517,728	62,036,455
Mar. 2013	1,295	3	38,818,830	25,291,838
Jan. 2015	825	2	34,547,902	30,485,484
Nov. 2014 (0 d)	170	3	27,558,188	21,851,341
10 d	420	3	51,666,990	32,136,069
25 d	770	3	47,310,766	29,582,874
35 d	1,010	3	43,025,214	33,244,075
65 d	1,730	3	66,577,150	48,833,283

Fig. 2. Bud burst rate (%) of apple 'Fuji' terminal buds in forcing condition and chill unit at sampling date. (A) Samples collected from the trees in field conditions in 2012–13, (B) samples collected from the trees in field conditions in 2014–15, and (C) samples collected from the branches exposed to artificial chilling conditions. Chill units were shown in parentheses of each sample name.

Discussion

CHARACTERIZATION OF THE DORMANCY STATUS AND GENE EXPRESSION LEVELS IN TERMINAL FLOWER BUDS UNDER DIFFERENT ENVIRONMENTAL CONDITIONS. Quantitatively analyzing the state of bud dormancy is very challenging, and there are still few molecular and physiological markers suitable for evaluating the bud endodormancy state. The bud burst rate under forcing conditions has traditionally been used to predict the endodormancy status. In addition, the accumulated CU is useful for evaluating endodormancy progression. Thus, we analyzed the bud burst rate and the CU at several time points during the endodormancy period in the field and under artificial chilling conditions (Fig. 2). As expected, the bud burst rate gradually increased with increasing CU. However, the bud burst rate decreased in response to a chilling treatment and did not reach 100% even after a normally sufficient exposure to chilling conditions. We suspected that some stresses associated with the prolonged chilling of detached branches contributed to the decrease in bud burst rate. It is possible that the buds exposed to continuous chilling stress may require more extensive chilling for dormancy release compared with buds exposed to chilling conditions in the field. If this is the case, the exact CR may vary considerably depending on environmental conditions. Further studies will be required to test this hypothesis.

Our cluster analysis revealed that the genes differentially expressed under natural chilling conditions did not exhibit global expression-level changes in response to continuous chilling, and vice versa (Fig. 3). This suggested that the global expression patterns differed between the field-grown naturally chilled buds and the continuously chilled buds, even though they were exposed to similar amounts of accumulated chilling and expected to have a similar dormancy status. Comparing samples from more than one environmental background can easily lead to the exclusion of genes affected by specific environmental changes. This results in the detection of genes related to a specific endogenous status (e.g., dormancy status) but whose expression-level changes are unaffected by specific environmental conditions. Nevertheless, some GO terms, such as “oxidation-reduction process” and “iron ion binding,” were enriched in clusters 2 and 7 (Table 2), which comprised genes whose expression levels were up-regulated during prolonged chilling under continuous and natural chilling conditions, respectively. This result is consistent with the previous finding that oxidative stress and oxidation-reduction pathways are associated with prolonged chilling-induced physiological

Fig. 3. Hierarchical clustering of the expressed genes in apple dormant buds. Chill units were shown in parentheses of each sample name. Eight clusters were constructed.

a BLAST search and five genes encoded transcription factors. The annotations indicated that *MD09G1146000*, *MD09G1009100*, *MD14G1126900*, *MD16G1140800*, and *MD15G1369700* encode PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), MADS-BOX-LIKE, MYC2-LIKE, APETALLA2/ETHYLENE-RESPONSIVE 113 (ERF113), and ODORANT1 (ODO1) transcription factors, respectively. First four genes and the last one exhibited considerably up-regulated and down-regulated expression, respectively, in response to the chilling treatment (Fig. 4). Among five transcription factor genes, *MADS-BOX-LIKE* and *ODO1* fell into cluster 2 and cluster 7, respectively, suggesting that expression changes of these genes may be related to oxidative stress. Other three genes were not included in cluster analysis (data not shown). As for *MADS-BOX-LIKE*, our phylogenetic tree constructed using *MD09G1009100* and *FLC* clade genes in arabidopsis, grape, peach, and poplar indicated that *MD09G1009100* encodes a MADS-box transcription factor belonging to the *FLC* clade (Fig. 5), even though it was annotated as a truncated CAULIFLOWER D-like transcription factor in the apple genome database. The results of a BLAST search suggested this gene was highly similar to the Japanese pear *FLC*-like gene sequence (accession no. AB524589; $e = 1 \times 10^{-23}$). Most *DAM* genes including *MdDAM1–4* were not included in our DEG list and also in GO analysis because of either lower RPKM value or a lower CV score.

Table 2. Significantly enriched gene ontology (GO) terms in clusters 1, 2, 4, and 7 constructed by cluster analysis (Fig. 3) of apple dormant buds RNA-Seq reads.

Cluster	GO ID	numDEInCat ^z	numInCat ^y	GO term	Ontology ^x	FDR ^w
cl.1	GO:0015074	8	78	DNA integration	BP	0
	GO:0003676	20	1177	Nucleic acid binding	MF	1.31E-11
cl.2	GO:0008171	6	73	O-methyltransferase activity	MF	1.34E-04
	GO:0009058	8	242	Biosynthetic process	BP	1.34E-04
	GO:0020037	11	657	Heme binding	MF	1.71E-03
	GO:0008152	15	1375	Metabolic process	BP	1.71E-03
	GO:0009055	11	747	Electron carrier activity	MF	4.54E-03
	GO:0055114	18	2123	Oxidation-reduction process	BP	5.17E-03
	GO:0005506	9	639	Iron ion binding	MF	1.76E-02
	GO:0003824	14	1885	Catalytic activity	MF	6.07E-02
cl.4	GO:0006334	23	106	Nucleosome assembly	BP	6.29E-22
	GO:0000786	22	101	Nucleosome	CC	2.11E-21
	GO:0046982	22	134	Protein heterodimerization activity	MF	1.23E-18
	GO:0006270	11	19	DNA replication initiation	BP	4.07E-14
	GO:0005634	49	1016	Nucleus	CC	4.81E-14
	GO:0008017	20	128	Microtubule binding	MF	1.62E-13
	GO:0006260	18	100	DNA replication	BP	1.62E-13
	GO:0042555	10	17	MCM complex	CC	4.04E-13
	GO:0005871	18	110	Kinesin complex	CC	1.37E-12
	GO:0003777	18	111	Microtubule motor activity	MF	1.37E-12
	GO:0007018	18	111	Microtubule-based movement	BP	1.37E-12
	GO:0003678	10	22	DNA helicase activity	MF	6.17E-12
	GO:0003677	62	1973	DNA binding	MF	6.71E-11
	GO:0051726	10	29	Regulation of cell cycle	BP	2.49E-10
	GO:0000079	10	48	Regulation of cyclin-dependent protein serine/threonine kinase activity	BP	3.49E-08
	GO:0019901	10	48	Protein kinase binding	MF	3.49E-08
	GO:0005576	11	80	Extracellular region	CC	4.98E-08
	GO:0009664	8	44	Plant-type cell wall organization	BP	1.46E-06
	GO:0005874	7	41	Microtubule	CC	5.43E-05
	GO:0005200	5	36	Structural constituent of cytoskeleton	MF	4.48E-03
	GO:0043234	5	33	Protein complex	CC	4.48E-03
	GO:0051258	5	33	Protein polymerization	BP	4.48E-03
	GO:0007017	5	48	Microtubule-based process	BP	1.63E-02
	GO:0005524	68	3470	ATP binding	MF	1.76E-02
	GO:0015979	6	91	Photosynthesis	BP	1.85E-02
	GO:0005975	19	739	Carbohydrate metabolic process	BP	8.90E-02
cl.7	GO:0048046	12	80	Apoplast	CC	1.13E-07
	GO:0046274	8	47	Lignin catabolic process	BP	7.58E-06
	GO:0052716	8	47	Hydroquinone:oxygen oxidoreductase activity	MF	7.58E-06
	GO:0055114	52	2123	Oxidation-reduction process	BP	4.24E-05
	GO:0016165	5	26	Linoleate 13S-lipoxygenase activity	MF	6.03E-05
	GO:0046872	24	767	Metal ion binding	MF	8.77E-05
	GO:0005507	14	222	Copper ion binding	MF	1.11E-04
	GO:0006629	16	330	Lipid metabolic process	BP	2.98E-04
	GO:0020037	24	657	Heme binding	MF	3.75E-04
	GO:0004601	11	162	Peroxidase activity	MF	1.39E-03
	GO:0006979	11	171	Response to oxidative stress	BP	2.79E-03
	GO:0005506	20	639	Iron ion binding	MF	6.94E-03
	GO:0016787	18	578	Hydrolase activity	MF	2.37E-02
	GO:0003700	21	765	Transcription factor activity, sequence-specific DNA binding	MF	2.61E-02
	GO:0005509	13	406	Calcium ion binding	MF	6.50E-02

^zNumber of differentially expressed genes included in this ontology.

^yNumber of total genes included in this ontology.

^xBP = biological process; MF = molecular function; CC = cellular component.

^wFalse discovery rate.

Table 3. Number of differentially expressed genes (false discovery rate <0.05) between endo- and ecodormant apple flower bud samples. Twelve pairwise comparisons were performed using four endo- and three ecodormant samples assigned based on bud burst rate in forcing condition (Fig. 2).

		Ecodormancy		
		Jan. 2013	Mar. 2013	Jan. 2015
Endodormancy	Oct. 2012	5,406	7,728	6,151
	Nov. 2012	16,698	10,313	8,176
	Nov. 2014 (0 d)	12,448	4,039	5,936
	10 d	20,488	8,906	4,802

Table 4. Gene annotations and Pearson correlation (r) between the gene expression value reads per kilobase of millions and the chill unit for the 44 genes differentially expressed between endo- and ecodormancy in terminal flower buds. Genes were annotated based on a BLAST search of the NCBI non-redundant (nr) database. The top matches are listed.

Gene	Annotation by nr	r
MD09G1146000	Transcription factor PIF4-like (<i>Malus ×domestica</i>)	0.86
MD11G1009500	F-box/kelch-repeat protein At2g44130-like (<i>M. ×domestica</i>)	0.85
MD07G1171200	Vacuolar amino acid transporter 1-like (<i>Pyrus ×bretschneideri</i>)	0.85
MD15G1267300	Anthocyanidin 3-O-glucosyltransferase 5-like isoform X2 (<i>M. ×domestica</i>)	0.85
MD04G1139000	Uncharacterized protein LOC103433525 (<i>M. ×domestica</i>)	0.84
MD09G1009100	Truncated transcription factor CAULIFLOWER D-like isoform X3 (<i>P. ×bretschneideri</i>)	0.79
MD12G1114200	UDP-glycosyltransferase 86A1-like (<i>M. ×domestica</i>)	0.76
MD14G1126900	Transcription factor MYC2-like (<i>M. ×domestica</i>)	0.72
MD06G1142700	SNF1-related protein kinase regulatory subunit gamma-1-like (<i>M. ×domestica</i>)	0.67
MD16G1140800	Ethylene-responsive transcription factor ERF113 (<i>M. ×domestica</i>)	0.66
MD13G1021100	HMG-Y-related protein A-like, partial (<i>P. ×bretschneideri</i>)	0.66
MD09G1141800	UDP-glycosyltransferase 71A16-like (<i>M. ×domestica</i>)	0.63
MD06G1173800	Probable inactive poly (ADP-ribose) polymerase SRO5 (<i>M. ×domestica</i>)	0.62
MD11G1009700	Probable polyamine transporter At3g13620 (<i>M. ×domestica</i>)	-0.60
MD15G1048500	Serine/threonine-protein kinase At5g01020-like (<i>M. ×domestica</i>)	-0.60
MD15G1248200	Uncharacterized protein LOC103436125 (<i>M. ×domestica</i>)	-0.60
MD03G1140400	Protein DMR6-like OXYGENASE 2-like isoform X1 (<i>P. ×bretschneideri</i>)	-0.62
MD12G1240300	Probable aldo-keto reductase 1 (<i>M. ×domestica</i>)	-0.62
MD07G1274500	Short-chain dehydrogenase TIC 32, chloroplastic-like (<i>P. ×bretschneideri</i>)	-0.62
MD13G1231500	Ubiquinol oxidase, mitochondrial-like (<i>M. ×domestica</i>)	-0.63
MD07G1071900	Glucosyltransferase 9-like (<i>P. ×bretschneideri</i>)	-0.63
MD13G1257800	4-Coumarate coenzyme A ligase 5 (<i>Eriobotrya japonica</i>)	-0.63
MD07G1043700	FAD-dependent urate hydroxylase-like (<i>M. ×domestica</i>)	-0.64
MD11G1074900	Vacuolar cation/proton exchanger 3-like (<i>M. ×domestica</i>)	-0.64
MD10G1321200	Peroxidase 42-like (<i>M. ×domestica</i>)	-0.64
MD13G1046900	Putative leucoanthocyanidin reductase (<i>M. ×domestica</i>)	-0.64
MD06G1211400	Leucoanthocyanidin reductase-like isoform X1 (<i>M. ×domestica</i>)	-0.64
MD15G1264200	Uncharacterized protein LOC103401168 isoform X2 (<i>M. ×domestica</i>)	-0.65
MD00G1088100	Caffeoyl-CoA O-methyltransferase isoform X2 (<i>M. ×domestica</i>)	-0.66
MD02G1172900	Uncharacterized protein LOC103418353 isoform X1 (<i>M. ×domestica</i>)	-0.66
MD02G1177100	Protein IRX15-like (<i>M. ×domestica</i>)	-0.66
MD05G1016200	Putative UDP-glucuronate:xylan alpha-glucuronosyltransferase 3 isoform X1 (<i>M. ×domestica</i>)	-0.66
MD15G1139000	Fatty acid amide hydrolase-like (<i>M. ×domestica</i>)	-0.67
MD08G1102300	UDP-glucuronate:xylan alpha-glucuronosyltransferase 2 isoform X1 (<i>M. ×domestica</i>)	-0.68
MD13G1055700	DNA-3-methyladenine glycosylase-like (<i>M. ×domestica</i>)	-0.69
MD00G1116600	GDSL esterase/lipase At5g37690 (<i>M. ×domestica</i>)	-0.69
MD11G1232700	(-)-Alpha-pinene synthase-like (<i>M. ×domestica</i>)	-0.69
MD17G1158300	Beta-amyrin synthase-like (<i>M. ×domestica</i>)	-0.69
MD15G1369700	Protein ODORANT1-like (<i>M. ×domestica</i>)	-0.71
MD00G1139900	Uncharacterized protein LOC103950116 isoform X1 (<i>P. ×bretschneideri</i>)	-0.72
MD11G1161800	Fatty acyl-CoA reductase 3-like (<i>P. ×bretschneideri</i>)	-0.74
MD15G1009100	GDSL esterase/lipase EXL3-like (<i>M. ×domestica</i>)	-0.76
MD15G1008800	GDSL esterase/lipase EXL3-like (<i>M. ×domestica</i>)	-0.78
MD11G1153800	GDSL esterase/lipase At5g22810-like (<i>M. ×domestica</i>)	-0.78

PIF4 = phytochrome-interacting factor 4.

changes, such as dormancy release (Bai et al., 2013, Khalil-Ur-Rehman et al., 2017, Liu et al., 2012), cold hardening or both (John et al., 2016).

IDENTIFICATION OF TRANSCRIPTION FACTOR GENES ASSOCIATED WITH ENODORMANCY RELEASE IN APPLE. Transcription factors have significant roles as master regulators of diverse plant traits (Becker and Theissen, 2003; Ng and Yanofsky, 2001). The aim of this study was to identify candidate master regulators of dormancy release and the CR, and transcription factors may be suitable for this role. Although most of the identified genes in DEG list encoded enzymes involved in metabolic processes,

Fig. 4. Expression patterns of five candidate transcription factor genes that were isolated from our screening strategy. These five genes were included in 44 differentially expressed genes (DEGs) differentially expressed between endodormancy and ecodormancy, with showing expression changes correlated with chill units. Expression patterns in field conditions in 2012–13 (A, C) and artificial chilling conditions (B, D). (A, B) Four DEGs up-regulated during dormancy release. (C, D) One DEG down-regulated during dormancy release. *FLOWERING LOCUS C-LIKE* (*FLC-LIKE*): MD09G1009100, *MYC2*: MD14G1126900, *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*): MD09G1146000, *APETALLA2/ETHYLENE-RESPONSIVE113* (*AP2/ERF113*): MD16G1140800, *ODORANT1* (*ODO1*): MD15G1369700. Error bars represent sd.

such as GDSL esterase/lipase, hydrolase and transporters (Table 3), five genes, *PIF4*, *MYC2*, *FLC-LIKE*, *AP2/ERF113*, and *ODO1*, encoded transcription factors. Among them, *PIF4* and *MYC2* were known to be related to phytohormone metabolism, whereas *AP2/ERF113* was involved in meristem activity. *PIF4* regulates auxin biosynthesis at high temperature (Franklin et al., 2011) and positively regulates growth and development at elevated temperature (Gangappa et al., 2017). *MYC2* is a master regulator of the response to the jasmonate in arabidopsis and is involved in jasmonic acid-regulated plant development, lateral adventitious root formation, flowering time, and shade avoidance syndrome (Goossens et al., 2015; Kazan and Manners, 2013). *AP2/ERF113* promotes shoot regeneration from calli (Che et al., 2006) and related to tissue repair in floral stem (Asahina et al., 2011). On the other hand, *ODO1* encodes R2R3-MYB transcription factor and regulates volatile benzenoid/phenylpropanoid biosynthesis pathway in petunia [*Petunia × hybrida* (Colquhoun and Clark, 2011)], suggesting that it might be involved in secondary metabolism than dormancy regulation. In fact, an anthocyanin biosynthesis-related gene, leucoanthocyanidin reductase gene, was also included in DEG list as with *ODO1*. To the best of our knowledge, none of the reports focused on *PIF4*, *MYC2*, and *AP2/ERF113*, regarding bud dormancy regulation so far, and

our findings suggested that auxin biosynthesis, jasmonic acid signaling, and meristem activity that are mediated by *PIF4*, *MYC2*, and *AP2/ERF113*, respectively, are associated with dormancy release in apple. Our identification of transcription factors involved in phytohormone metabolism and meristem activity is consistent with the previous report suggesting that phytohormone-related pathways and post-embryonic development may be involved in chilling-mediated dormancy release and fruit set in apple (Kumar et al., 2017).

As for *FLC-LIKE*, our results are consistent with the previous finding that an *FLC-LIKE* gene is differentially expressed during apple dormancy release (Porto et al., 2015). *MD09G1009100* was localized to the upper distal part of chromosome nine, which was recently indicated as being involved in the genetic control of flowering or vegetative budbreak (Allard et al., 2016; Celton et al., 2011; Urrestarazu et al., 2017; van Dyk et al., 2010). Significant *FLC* and *FLC-LIKE* gene expression changes during dormancy were also reported for chinese cherry [*Prunus pseudocerasus* (Zhu et al., 2015)], leafy spurge (Doğramaci et al., 2014), *Taihangia rupestris* (Du et al., 2008), and trifoliate orange [*Poncirus trifoliata* (Zhang et al., 2009)]. Kumar et al. (2016a) revealed that the expression levels of certain *FLC-LIKE* genes (*MDP0000186558* and *MDP0000218227*) are down-regulated

toward the end of the dormancy and budbreak stage. They also completed a genome-wide phylogenetic analysis of the apple MADS-box gene family and identified four putative *FLC-LIKE*

genes. *MD09G1009100* (MDP0000126259 and MDP0000167381 in the old version of the apple genome) was not included among the four genes probably because of a lack of a MADS-

domain in MDP0000126259 and MDP0000167381. Although we revealed the up-regulation of *MD09G1009100* expression during endodormancy release, we cannot rule out the possibility that it is down-regulated toward the end of the dormancy and budbreak stage as is the case for other *FLC-LIKE* genes (Kumar et al., 2016a). Indeed, *MD09G1009100* was down-regulated from January to March, specifically during the transition from the ecodormancy stage to the budbreak stage. So far, there have been inconsistencies regarding the presence and identification of apple *FLC-LIKE* genes in previous studies by different researchers, for example, Tian et al. (2015) did not detect any *FLC-LIKE* genes when they examined the apple MADS-box gene family. Thus, we identified apple *FLC-LIKE* genes using a recently updated version of the apple genome. A BLAST search of the apple genome using the MD09G1009100 amino acid sequence as a query identified six genes with high e values ($e < 1 \times 10^{-2}$). A phylogenetic analysis revealed that four of these genes belong to the *FLC* clade and two genes were included in the clade other than

Fig. 5. Phylogenetic tree consisting of *FLOWERING LOCUS C* (*FLC*) clade genes from arabidopsis (*FLC*, MAF1–5), grape (GSVIVT01033067001), peach (Prupe.3G307800.1), poplar (Potri001G328600, 001G058200, 003G169700, 003G169850, 003G170000, 003G170200), and apple (MD05G1037100, 09G1009100, 10G1041100, 17G1001300) and some MADS-box genes from arabidopsis. The tree was constructed with 1000 bootstrap replication using the MEGA7 program (Kumar et al., 2016b), followed by an amino acid sequence alignment using MAFFT (Katoh and Standley, 2013) and trimAL (Capella-Gutiérrez et al., 2009). The arrow indicates the position of *MD09G1009100*.

Fig. 6. Expression patterns of apple *FLOWERING LOCUS C-LIKE* (*FLC-LIKE*) gene, *MD09G1009100*, and three other *FLC-LIKE* genes, *MD05G1037100*, *MD10G1041100*, and *MD17G1001300*, under field conditions in 2012–13 (A) and under chilling treatment conditions (B). Error bars represent SD.

FLC (data not shown). As consequence, we identified four *FLC-LIKE* genes (*MD05G1037100*, *MD09G1009100*, *MD10G1041100*, and *MD17G1001300*) in apple genome. None of these genes correspond to the *FLC-LIKE* genes identified by Kumar et al. (2016a). The expression patterns of three identified *FLC-LIKE* genes, which differed from that of *MD09G1009100*, did not exhibit dormancy-related expression changes, suggesting these genes are likely unrelated to endodormancy release (Fig. 6). Additional studies focusing on the biological function of *MD09G1009100* will help to clarify its role during endodormancy and budbreak. In addition, because poor performance of budbreak often occurs in lateral vegetative buds than in terminal flower buds, the involvement of *MD09G1009100* on bud dormancy regulation in lateral parts should be focused in the future to solve the problem about budbreak disorder caused by global climate change.

In conclusion, our RNA-Seq analysis identified four transcription factor genes, *PIF4*, *FLC-LIKE*, *AP2/ERF113*, and *MYC2*, that were correlated with the CU and expressed differently between endodormant and ecodormant buds, suggesting that these transcription factors are closely associated with chilling-mediated endodormancy release in apple.

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