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Custom Microarray Analysis for Transcript Profiling of Dormant Vegetative Buds of Japanese Apricot during Prolonged Chilling Exposure

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Bud dormancy is a critical developmental process for perennial plant survival, and also an important physiological phase that affects the next season's growth of temperate fruit trees. Bud dormancy is regulated by multiple genetic factors, and affected by various environmental factors, tree age and vigor. To understand the molecular mechanism of bud dormancy in Japanese apricot (*Prunus mume* Sieb. et Zucc.), we constructed a custom oligo DNA microarray covering the Japanese apricot dormant bud ESTs referring to the peach (*P. persica*) genome sequence. Because endodormancy release is a chilling temperature-dependent physiological event, genes showing chilling-mediated differential expression patterns are candidates to control endodormancy release. Using the microarray constructed in this study, we monitored gene expression changes of dormant vegetative buds of Japanese apricot during prolonged artificial chilling exposure. In addition, we analyzed seasonal gene expression changes. Among the 58539 different unigene probes, 2345 and 1059 genes were identified as being more than two-fold up-regulated and down-regulated, respectively, following chilling exposure for 60 days ($P < 0.05$). Cluster analysis suggested that the expression of the genes showing expression changes by artificial chilling exposure were coordinately regulated by seasonal changes. The down-regulated genes included *P. mume DORMANCY-ASSOCIATED MADS-box* genes, which supported previous quantitative RT-PCR and EST analyses showing that these genes are repressed by prolonged chilling exposure. The genes encoding lipoxygenase were markedly up-regulated by prolonged chilling. Our parametric analysis of gene-set enrichment suggested that genes related to jasmonic acid (JA) and oxylipin biosynthesis and metabolic processes were significantly up-regulated by prolonged chilling, whereas genes related to circadian rhythm were significantly down-regulated. The results obtained from microarray analyses were verified by quantitative RT-PCR analysis of selected genes. Taken together, we have concluded that the microarray platform constructed in this study is applicable for deeper understanding of the molecular network related to agronomically important bud physiology, including dormancy release.

Key Words: bud dormancy, circadian rhythm, jasmonic acid, MADS-box, 454-pyrosequencing.

Introduction

Seasonal growth and developmental control allows woody perennials to synchronize annual growth and to avoid injury from environmental stresses such as cold in the winter. Dormancy is one such controlling mechanisms that enable woody perennials to adapt to seasonal climate changes. Furthermore, dormancy is an agronomically important trait, influencing fruit production in temperate woody deciduous perennials by promoting survival during unfavorable climates, and affecting flowering and subsequent vegetative growth in the following growing season. Additionally, recent global climate changes, such as global warming, are reported to affect the dormancy release of fruit trees (Sugiura et al., 2007). Therefore, it is necessary to investigate the genetic factors underlying the control of dormancy. Elucidation of the molecular basis of dormancy regulation in temperate fruit tree spe-

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cies could enable artificial control of dormancy through cultural practices, and will be useful for rapid breeding techniques such as marker-assisted selection.

The seasonal activity-dormancy phase transition of vegetative growth of Japanese apricot (*Prunus mume* Sieb. et Zucc.) occurs gradually, and as with other temperate fruit trees, takes a long time. Although flowering is often observed from February to March under field conditions in Kyoto, vegetative bud flushing does not occur until April. Shoot growth cessation of long shoots is observed from June, and the majority of long shoots have stopped active growth by August; trees shed their leaves by early December. When branches cut from trees are incubated in forcing conditions, bud burst of long branches collected in early June is observed. Bud burst then becomes unstable, and fluctuates depending on shoots and years when collected after late June and during summer. This suggests that these buds are facultatively dormant (Yamane et al., 2008). In fact, a second flushing of 'Nanko' trees under field conditions during summer suggests that they are not completely dormant. However, bud burst has never been observed in long branches collected in autumn in any of our forcing conditions (Sasaki et al., 2011; Yamane et al., 2008), suggesting that these buds are constitutive dormant. Bud dormancy can be considered as the inability of a meristem to resume growth under favorable conditions (Rohde and Bhalerao, 2007). Lang (1987) and Lang et al. (1987) gave definitions of dormancy as being paradormancy, endodormancy, and ecodormancy. Both endodormancy and paradormancy can be defined as a state induced by the perception of the promoting environmental or endogenous signaling cue, whether this originated solely within the meristem-containing tissue (endodormant), or in a structure distinct from the structure undergoing dormancy (paradormant). Until the cessation of shoot growth, axillary buds were undoubtedly in a paradormant state and unable to grow because of apical dominance; however, after cessation of growth until defoliation, axillary or lateral buds were neither defined as paradormant or endodormant in Japanese apricot because the factors making the buds remain in the dormant state are unclear. Although environmental or endogenous cues involved in the transition from paradormancy to endodormancy are not well known, a specific amount of chilling exposure is known to critically induce the shift of endodormancy to ecodormancy. Ecodormancy is a state brought about by the limitation of growth-promoting factors such as water and nutrients. In Japanese apricot, the flower and vegetative buds of long branches of 'Nanko' shift from endodormant to ecodormant during December and January, respectively (Sasaki et al., 2011; Yamane et al., 2006, 2008).

Bud phenology during dormancy consists of different morphological and physiological events; these include bud scale initiation, endodormancy, ecodormancy, bud burst, and bud flushing. Of these, endodormancy release is affected mainly by genotypically determined chilling requirements, whereas bud flushing is affected mainly by a heat requirement following chilling requirement fulfillment. All these events are influenced by various environmental factors, as well as by tree age and vigor (Cooke et al., 2012; Rohde and Bharelao, 2007). Recent genetic analysis conducted in *Prunus* fruit species, including almond (*P. dulcis*) (Sánchez-Pérez et al., 2012), apricot (*P. armeniaca*) (Campoy et al., 2011; Olukolu et al., 2009), and peach (*P. persica*) (Fan et al., 2010), demonstrated that both the chilling requirement and timing of blooming in the field showed quantitative inheritance. These studies also identified various quantitative trait loci (QTLs) affecting these traits. These findings suggested that bud dormancy of Japanese apricot is also controlled by numerous genes. A recent proteomic analysis has revealed that many protein spots show differential expression among different dormant stages of Japanese apricot flower buds (Zhuang et al., 2013).

Yamane et al. (2008) searched for genes up-regulated during endodormancy using the RNA subtraction technique and revealed that *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes are up-regulated during the induction of endodormancy, and down-regulated during endodormancy release (Yamane et al., 2008). Japanese apricot *DAM*s (*PmDAM1–PmDAM6*) were found to be arranged in tandem arrays in the genome of Japanese apricot (Sasaki et al., 2011). Expressional and transgenic analyses suggested that PmDAMs are candidates that play a crucial role in the control of bud dormancy in Japanese apricot (Sasaki et al., 2011; Yamane et al., 2008). Furthermore, information is available for other genes that have altered dormancy of *Prunus*, such as *Populus FLOWERING LOCUS T* homolog (Srinivasan et al., 2011). This suggests that genes in addition to *PmDAM*s are expected to be involved in bud dormancy regulation of Japanese apricot. However, the whole picture of the molecular basis of dormancy regulation in Japanese apricot is still far from clear.

Large-scale measurements of gene expression using microarrays have proven useful in the characterization of molecular networks during dormancy in various plant species including sessile oak [*Quercus petraea* (Matt.) Liebl.] (Derory et al., 2006), poplar (*Populus* spp.) (Ko et al., 2011; Ruttink et al., 2007), raspberry (*Rubus idaeus* L.) (Mazzitelli et al., 2007), grapevine (*Vitis riparia* Michx.) (Mathiason et al., 2009), blackcurrant (*Ribes nigrum* L.) (Hedley et al., 2010), and leafy spurge (*Euphobia esula* L.) (Doğramaci et al., 2010). A substantial amount of target transcriptome is required for microarray-based gene expression analysis. Previously, we obtained expressed sequence tag (EST) information from Japanese apricot buds at various dormant phases using 454-pyrosequencing (Habu et al., 2012). As reported in other organisms, sequences assembled from the 454-pyrosequencing reads were useful for designing probe sequences of microarrays (Bellin et al., 2009; Zenoni et al., 2011), and as reference sequences for short-read sequencing (Fraser

et al., 2011). As 454-pyrosequencing has proven very useful for genome and transcriptome analyses, we attempted in this study to construct a custom microarray for the transcript profiling of Japanese apricot dormant buds. To search for candidate genes involved in chilling requirement fulfillment and endodormancy release, we performed microarray and statistical analysis to identify genes showing expression changes during prolonged artificial chilling exposure. Microarray analysis was also used to monitor seasonal expression changes of the selected genes. These analyses provide further our understanding of the dynamic molecular network regulating Japanese apricot bud dormancy.

Materials and Methods

Construction of a custom 180-K microarray for transcript profiling of Japanese apricot dormant buds

We previously obtained Japanese apricot bud ESTs consisting of 28382 contigs and 85247 singletons from vegetative and flower buds at 10 different dormant stages (Habu et al., 2012). The 113629 unigenes obtained were used for microarray probe selection using the Agilent eArray application tool (https://earray.chem.agilent.com/earray/, August 1, 2013) following the manufacturer's instruction manual. We constructed 60K probes with a length of 60 bases. We took into consideration lower probe redundancy and increased coverage of probes to the whole expressed genes in Japanese apricot dormant buds by using trial-and-error approaches (as described in the Results section).

RNA extraction and array hybridization

Field-grown Japanese apricot 'Nanko' trees (11 years old, seed-grafted) grown at Kyoto University Experimental Farm, Kyoto, Japan, were used in this study. Bud samples for the following two datasets were collected. One-year-old long branches (> 50 cm) were collected from three different trees in mid-November 2008, and used as biological replicates (Dataset 1: chilling treatment). Collected branches were artificially defoliated and transferred to a growth chamber under a 9-h light/15-h dark photoperiod cycle at temperature conditions of 12°C in the light and 6°C in the dark. The basal parts of these branch cuttings were placed in water. Vegetative buds were excised from the middle portions of the branches 0, 40, and 60 days after treatment, and immediately frozen in liquid nitrogen and stored at −80°C until required. Sixty days of chilling treatment were enough for vegetative buds of 'Nanko' to fulfill the chilling requirements, whereas 0, and 40 days were insufficient (Sasaki et al., 2011).

One-year-old long branches were collected monthly, in the middle of each month, from June 2008 to March 2009 (Dataset 2: seasonal change). Vegetative buds were excised from the middle portions of the branches, immediately frozen in liquid nitrogen, and stored at −80°C until required. Vegetative buds of 'Nanko' long branches from July to January were considered to be endodormant based on previous reports (Sasaki et al., 2011; Yamane et al., 2008), even though the buds collected in these months include both facultatively and constitutively endodormant buds (see Introduction). The buds from June were considered to be dormant due to apical dominance, whereas buds from February and March were ecodormant.

Total RNA was isolated from the buds as described by Yamane et al. (2008). RNA concentration was measured by spectrophotometry (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA, USA) and integrity was assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). mRNA was labeled using the Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies) from total RNA (200 ng) according to the manufacturer's instructions. The labeled samples were hybridized to the oligoarray slides using a Gene Expression Hybridization Kit (Agilent Technologies) at 65°C for 17 h in an oven-chamber. Following hybridization, arrays were washed with Gene Expression Wash Buffer (Agilent Technologies) according to the manufacturer's instructions. The dried slides were scanned using an Agilent scanner. Raw data from scanning of the array were captured using Agilent Feature Extraction Ver. 9.5.1 (Agilent Technologies).

Microarray data analysis

Data sets for each array were normalized and analyzed using the Subio software platform (Subio Inc. Kagoshima, Japan). First, probes with low hybridization signals were removed. Then, hybridization intensities were $\log_2$ transformed, and arrays were normalized by global normalization, quantile normalization, and centering. For the samples in dataset 1 (three different time points during chilling treatment), three technical replicates (60K × 3 per sample) with three biological replicates were averaged, whereas three technical replicates were averaged for the samples in dataset 2 (10 different seasonal time points). *T*-tests were performed ($P < 0.05$) to identify probes with significant differential expression between each treatment in dataset 1. Cluster analysis of the expression patterns for differentially expressed genes was performed by Spearman correlation.

Analysis of Gene Ontology (GO) terms

Japanese apricot unigenes corresponding to each probe loaded onto our constructed microarray were annotated using BLASTX (Altschul et al., 1990) against the non-redundant protein database (nr) from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/, August 1, 2013), the *Arabidopsis* protein database (TAIR9) (http://www.arabidopsis.org/Blast/index.jsp, August 1, 2013), and peach (*Prunus persica*) genome database (v1) (http://www.rosaceae.org/species/prunus_persica/genome_v1.0, August 1, 2013) (The International Peach Genome Initiative, 2013)

Table 1. Primer sequences used for qRT-PCR.

Primer name	Sequence (5' to 3')
PmLOX2(ppa001631)-F	TTTGAAGAGGCTGGAAGGAA
PmLOX2(ppa001631)-R	GACCCCAGGTGTTGAGAATG
PmLOX4(ppa001112)-F	CGAAAGGAGGAACAGTGACC
PmLOX4(ppa001112)-R	GGCACACCCCTACAAGTGAT
PmPRR5(ppa002188)-F	ATTCTCATCGGTCCATCCAA
PmPRR5(ppa002188)-R	TTATTCTGGGACGCTGCTCT
PmFKF1(ppa002863)-F	GGCTGCATTCTCCTTCTCAG
PmFKF1(ppa002863)-R	TGACCCCAAGCAAATTTAGG
PmTUB-F	GTGTTGCCGAGGTGTTTTCT
PmTUB-R	CTCCTTCATCGCCATCATCT
PmUBQ-F	CGAACCCTAGCCGATTACAA
PmUBQ-R	AGTGGTTCGCCATGAAAGTC

using an E-value cutoff set at $1 \times e^{-5}$. For the GO annotation, Blast2GO software v2.4.8 (http://www.blast2go.org, August 1, 2013) (Conesa et al., 2005) was used with the nr annotations. Log_2 transformed values of fold changes among the signals from the dataset 1 samples and the TAIR9 annotations were used for the parametric analysis of gene set enrichment (PAGE) (Kim and Volsky, 2005) conducted on agriGO (Du et al., 2010) (http://bioinfo.cau.edu.cn/agriGO/, August 1, 2013) web-based software using a *P*-value cutoff of 0.05.

Quantitative RT-PCR (qRT-PCR) analysis

For genes that showed differential expression among the samples selected by microarray and statistical analysis, qRT-PCR was performed using LightCycler 480 (Roche, Basel, Switzerland), SYBR Green Master Mix (Roche), and gene-specific primers (Table 1). For primer design, the Japanese apricot EST database (JADB; Habu et al., 2012) and the Primer3 web interface (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, August 1, 2013) (Rozen and Skaletsky, 2000) were used. The reaction mixture consisted of 1 × SYBR master mix, 500 nM each of forward and reverse primers, and cDNA equivalent to 4 ng of total RNA, in 20-μL reaction volumes. PCR was performed using a program of 45 cycles at 95°C for 10 s, 57°C for 10 s, and 72°C for 20 s, with initial heating at 95°C for 5 min. Dissociation curve analysis was performed to confirm that fluorescence was only derived from gene-specific amplification. As a reference, the accumulation of Japanese apricot *TUBULIN* (*PmTUB*) transcript was monitored; ESTs corresponding to *PmTUB* were highly accumulated in paradormant, endodormant, and ecodormant buds at relatively similar levels according to the JADB (Habu et al., 2012). In addition, Japanese apricot *UBIQUITIN* (*PmUBQ*) (Sasaki et al., 2011) was used as a reference.

Results

Custom oligoarray construction

A total of 113629 unigenes previously obtained by RNAseq analysis of Japanese apricot dormant buds (Habu et al., 2012) were subjected to an Agilent eArray to extract candidate probes for a custom 180K (60K × 3 replicates) array. Among the 113629 unigenes, 93666 were selected as 60 mer oligonucleotide probe candidates. Next, redundant probes mapped to the same peach gene locus were removed. Finally, we selected probes to cover the 113629 unigenes putatively expressed in Japanese apricot dormant buds as much as possible; this resulted in 58539 probes being selected. A further 88 probes, corresponding to peach genes were designed using the Agilent eArray. These were derived from the peach database (http://www.rosaceae.org/gb/gbrowse/prunus_persica/, August 1, 2013) (The International Peach Genome Initiative, 2013), and included peach MADS-box genes, circadian clock related genes, and genes involved in chromatin modification. Consequently, 58627 probes (58539 probes of Japanese apricot +88 probes of peach) were selected for the microarray construction. These probes were mapped to all 12 peach scaffolds without any biased regions (Fig. 1), and matched to 17977 putative peach mRNAs predicted by whole-genome peach sequencing, which covered 62.7% of peach putative mRNAs. Among the 58627 probes, 26008 probes were annotated in the TAIR9 database (E-value cutoff = $1.0 \times e^{-5}$); these annotated probes covered genes involved in various biological processes (data not shown). Three replicates of each probe [(58539 + 88) × 3] were loaded onto the 180K array. In addition, fifteen probes corresponding to Japanese apricot bud ESTs detected at higher levels were selected as control probes, and ten replicates of each were loaded onto the array.

Extraction of differentially expressed genes (DEGs) by chilling treatment in dormant buds

In dataset 1 (chilling treatment), 5345 and 4781 genes were found to be significantly up-regulated (> 2-fold) following 60 d chilling treatment in comparison to 0 d and 40 d chilling treatments, respectively ($P < 0.05$). Among these, 2345 genes were significantly up-regulated in both data comparisons. Conversely, 3489 and 3213 genes were significantly down-regulated (> 2-fold) following 60 d chilling treatment when compared with 0 d and 40 d treatments, respectively. Among these, 1059 genes were significantly down-regulated in both data comparisons. The 2345 up-regulated and 1059 down-regulated genes identified in these comparisons were ranked based on the fold changes of signal intensities at 60 d versus 0 d of chilling treatment. Probes up-regulated more than 10-fold are listed in Table 2. The two probes with the greatest expression changes, Ume31635 and Ume13003, corresponded to putative homologs of the *Arabidopsis* genes At3g45140 and At1g72520, respectively. These encode lipoxygenase (LOX) genes, namely LOX2 and LOX4. LOXs catalyze the conversion of polyunsaturated fatty acids (lipids) into conjugated hydroperoxides, a major step in fatty acid degradation. In addition, LOXs are known to be involved in response to biotic and abiotic

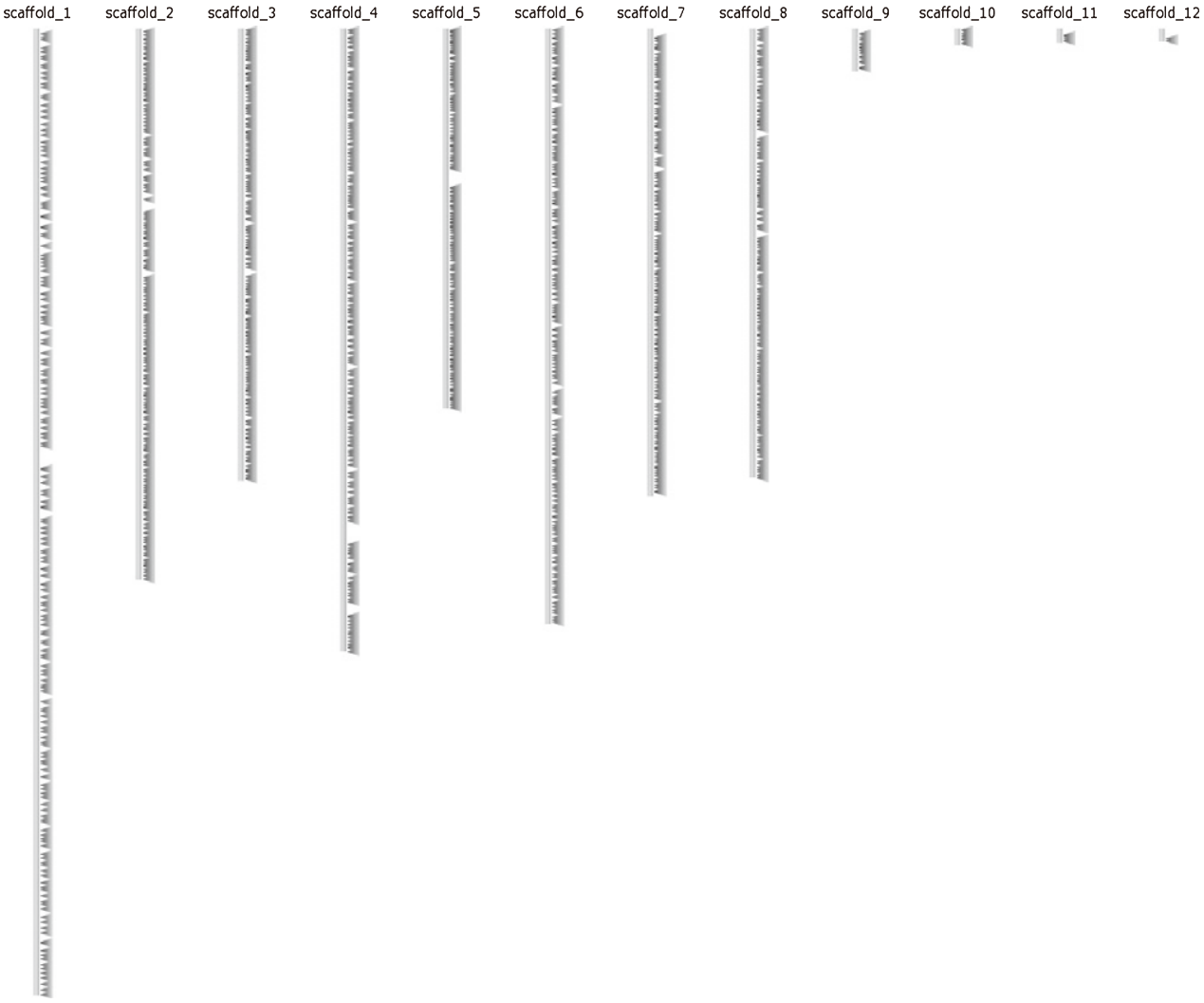

Fig. 1. Probes designed for the Japanese apricot microarray for transcriptomic analysis of dormant buds covered whole regions of the peach genome without any biased regions. The mapped regions of the ESTs, corresponding to the 58627 designed probes in 12 scaffolds of the peach genome (v1), are shown by arrowheads.

stresses, and the jasmonic acid (JA) biochemical pathways (Andreou and Feussner, 2009). Probes corresponding to genes down-regulated more than 10-fold are listed in Table 3. *Prunus mume DORMANCY ASSOCIATED MADS-box6* (*PmDAM6*) was one of the genes identified as being highly down-regulated gene under prolonged chilling treatment. Other probes that were annotated as *DAM*s, including *DAM1*, *3*, and *5* were also identified as being significantly down-regulated in chilling treatment (Table 3). When expression changes of all 20 *DAM* annotated probes were focused on, they were down-regulated by chilling exposure (Fig. 2A). They were also down-regulated from autumn to spring (Fig. 2B). The original data obtained in this study are accessible through GEO Series accession number GSE49368 in NCBI's Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/, August 1, 2013).

Cluster analysis of 2345 up-regulated genes by prolonged chilling suggested that the monthly bud samples fell into three groups (Fig. 3A). Similarly, cluster analysis of the 1059 down-regulated genes suggested the same grouping classifications for the monthly buds (Fig. 3B). Group 1 contained bud samples collected from June to October, group 2 contained those from November to January, and group 3 contained those from February and March. Many of the probes that were up-regulated by prolonged chilling tended to be down-regulated in group 2, whereas the majority of the probes down-regulated by prolonged chilling were up-regulated in group 2.

The genes up-regulated by prolonged chilling could be divided into two major categories (Fig. 3A). Most genes in category (a) were down-regulated in the group 1 months and up-regulated in March, whereas most genes in category (b) were up-regulated in the group 1 months and down-regulated in March. The genes down-regulated by prolonged chilling could also be divided into two major categories (Fig. 3B). Most genes in category (c) were down-regulated in the group 1 and 3 months, whereas

Table 2. Genes that were up-regulated in both 0 d to 60 d and 40 d to 60 d cold treatments (more than 10-fold up-regulated in 0 d to 60 d from the top in descending order).

measurement ID	0 d to 60 d		40 d to 60 d		Target_GB_Description	TAIR9_AGI	TAIR9_Description	TAIR9 *E*-value
	Fold Change	*P*-Value	Fold Change	*P*-Value				
Ume31635	604.52	5.16E-14	19.76	5.04E-13	Prunus dulcis clone Pdbcs-L14 putative lipoxigenase mRNA, partial cds	AT3G45140.1	LOX2(LIPOXYGENASE 2); lipoxygenase	6.00E-17
Ume13003	118.65	2.65E-19	8.56	3.03E-13	Prunus persica lipoxygenase 1 mRNA, complete cds	AT1G72520.1	lipoxygenase, putative	5.00E-11
Ume24114	103.46	4.15E-14	18.56	1.31E-11	Prunus dulcis clone Pdbcs-E45 putative carbonic anhydrase mRNA, partial cds	AT3G01500.3	CA1 (CARBONIC ANHYDRASE 1); carbonate dehydratase/zinc ion binding	9.00E-17
Ume30537	73.24	1.87E-19	8.89	1.86E-10	No hits found	AT5G44360.1	FAD-binding domain-containing protein	7.00E-09
Ume19612	61.78	5.55E-17	63.50	2.24E-18	Stevia rebaudiana UDP-glycosyltransferase 85A8 mRNA, complete cds	AT1G22360.1	AtUGT85A2 (UDP-glucosyl transferase 85A2)	4.00E-14
Ume19411	57.38	7.86E-20	12.46	1.07E-10	Fragaria vesca subsp. americana clone fosmid 41O22, complete sequence	AT1G61680.1	TPS14 (TERPENE SYNTHASE 14); S-linalool synthase	2.00E-13
Ume14286	47.60	2.35E-16	32.96	1.72E-13	Ricinus communis cytochrome P450, putative, mRNA	AT4G15300.1	CYP702A2; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding	2.00E-08
Ume21509	40.46	8.24E-11	40.62	5.94E-09	Populus trichocarpa predicted protein, mRNA	AT2G31690.1	lipase class 3 family protein	2.00E-08
Ume20942	34.98	1.23E-23	13.08	6.01E-14	Prunus salicina putative auxin-binding protein 2 (ABP2) mRNA, complete cds	AT1G72610.1	GER1 (GERMIN-LIKE PROTEIN 1); oxalate oxidase	2.00E-08
Ume55139	33.35	1.28E-13	3.2600	8.17E-13	PREDICTED: Vitis vinifera hypothetical protein LOC100257811 (LOC100257811), mRNA	AT3G29320.1	glucan phosphorylase, putative	5.00E-37
Ume12375	28.92	3.22E-13	21.32	1.92E-10	Medicago truncatula chromosome 8 clone mth2-145p10, complete sequence	AT2G38750.1	ANNAT4 (ANNEXIN ARABIDOPSIS 4); calcium ion binding/calcium-dependent phospholipid binding	1.00E-07
Ume22402	25.61	5.55E-18	3.93	2.30E-11	PREDICTED: Vitis vinifera hypothetical protein LOC100257811 (LOC100257811), mRNA	AT3G29320.1	glucan phosphorylase, putative	3.00E-43
Ume27874	24.57	2.97E-16	6.22	8.13E-16	Populus trichocarpa clone WS01212_G05 unknown mRNA	AT1G56630.1	triacylglycerol lipase	9.00E-32
Ume10139	23.16	2.46E-15	25.76	1.57E-18	Ricinus communis UDP-glucuronosyltransferase, putative, mRNA	AT1G22340.1	AtUGT85A7 (UDP-glucosyl transferase 85A7)	5.00E-12
Ume22074	22.80	3.85E-16	4.63	4.75E-11	Pyrus pyrifolia class V chitinase mRNA, complete cds	AT4G19750.1	glycosyl hydrolase family 18 protein	1.00E-16
Ume11754	20.83	8.18E-14	3.62	1.55E-10	PREDICTED: Vitis vinifera hypothetical protein LOC100257811 (LOC100257811), mRNA	AT3G29320.1	glucan phosphorylase, putative	4.00E-50
Ume30438	19.40	2.41E-09	30.39	5.94E-11	Prunus avium DREB1-like gene for dehydration responsive element binding protein 1 like protein, complete cds, clone:D2A	AT4G25490.1	CBF1 (C-REPEAT/DRE BINDING FACTOR 1); DNA binding/transcription activator/transcription factor	5.00E-06
Ume22940	19.32	2.11E-11	5.89	5.30E-08	Ricinus communis Polygalacturonase precursor, putative, mRNA	AT1G02460.1	glycoside hydrolase family 28 protein/polygalacturonase (pectinase) family protein	1.00E-19
Ume12800	18.50	3.01E-17	16.82	1.20E-13	Medicago truncatula chromosome 8 clone mth2-145p10, complete sequence	AT2G38750.1	ANNAT4 (ANNEXIN ARABIDOPSIS 4); calcium ion binding/calcium-dependent phospholipid binding	1.00E-07
Ume21598	17.78	2.54E-12	4.35	1.00E-08	Populus trichocarpa predicted protein, mRNA	AT5G64810.1	WRKY51; transcription factor	2.00E-28
Ume27770	17.50	1.41E-12	10.78	1.46E-13	Candida glabrata CBS138, CAGL0G02409g partial mRNA	AT4G30450.1	glycine-rich protein	1.00E-07
Ume57830	17.41	6.69E-14	18.00	1.95E-15	Lotus japonicus genomic DNA, chromosome 1, clone: LjT43J08, TM1255, complete sequence	AT4G12110.1	SMO1-1 (STEROL-4ALPHA-METHYL OXIDASE 1-1)	2.00E-36
Ume17307	16.65	5.73E-12	4.03	4.27E-11	Soybean clone JCVI-FLGm-1J17 unknown mRNA	AT1G26945.1	KDR (KIDARI); transcription regulator	3.00E-30
Ume20039	16.19	8.99E-13	4.44	3.34E-11	PREDICTED: Vitis vinifera hypothetical protein LOC100260291 (LOC100260291), mRNA	AT5G06720.1	peroxidase, putative	1.00E-13
Ume19991	15.36	1.73E-17	10.93	5.61E-14	Oryza sativa (japonica cultivar-group) Os01g0913600 (Os01g0913600) mRNA, complete cds	AT3G07880.1	Rho GDP-dissociation inhibitor family protein	4.00E-28
Ume58084	15.17	1.46E-09	13.65	4.58E-09	PREDICTED: Vitis vinifera hypothetical protein LOC100251646 (LOC100251646), mRNA	AT4G16230.1	carboxylesterase/hydrolase, acting on ester bonds	9.00E-14
Ume27557	14.81	1.36E-09	5.13	1.09E-12	No hits found	AT1G47980.1	unknown protein	8.00E-06
Ume09166	14.25	9.22E-09	4.67	6.99E-08	Lotus japonicus genomic DNA, chromosome 3, clone: LjT46B01, TM0116, complete sequence	AT2G38320.1	unknown protein	5.00E-30
Ume22365	13.96	1.53E-15	5.12	6.69E-11	PREDICTED: Vitis vinifera hypothetical protein LOC100250342 (LOC100250342), mRNA	AT3G14850.1	unknown protein	5.00E-32
Ume26820	13.88	1.50E-17	5.43	5.33E-11	Vitis pseudoreticulata glyoxal oxidase mRNA, complete cds	AT3G53950.1	glyoxal oxidase-related	4.00E-33
Ume45257	13.58	6.64E-15	6.48	3.04E-07	No hits found	AT1G65890.1	AAE12 (ACYL ACTIVATING ENZYME 12); catalytic	6.00E-11
Ume13929	13.56	1.05E-12	6.65	1.46E-11	No hits found	AT1G65890.1	AAE12 (ACYL ACTIVATING ENZYME 12); catalytic	4.00E-11
Ume50328	13.35	1.90E-11	15.23	9.93E-14	PREDICTED: Vitis vinifera hypothetical protein LOC100250551 (LOC100250551), mRNA	AT3G52450.1	PUB22 (PLANT U-BOX 22); ubiquitin-protein ligase	3.00E-28
Ume48679	13.04	1.11E-11	7.82	6.64E-12	Glycine max cDNA, clone: GMFL01-17-P07	AT5G64260.1	EXL2 (EXORDIUM LIKE 2)	4.00E-34
Ume46074	12.94	2.83E-11	16.10	1.79E-13	PREDICTED: Vitis vinifera hypothetical protein LOC100250551 (LOC100250551), mRNA	AT3G52450.1	PUB22 (PLANT U-BOX 22); ubiquitin-protein ligase	3.00E-28
Ume09558	12.65	8.82E-09	3.85	4.35E-03	Populus trichocarpa clone POP072-N07, complete sequence	AT1G11925.1	Encodes a Stigma-specific Stig1 family protein	5.00E-10
Ume10865	12.50	6.76E-11	7.90	1.02E-10	Populus trichocarpa cellulose synthase, mRNA	AT5G17420.1	IRX3 (IRREGULAR XYLEM 3); cellulose synthase	1.00E-18
Ume56350	12.41	6.79E-16	2.94	2.51E-11	Populus trichocarpa clone WS01212_G05 unknown mRNA	AT1G56630.1	triacylglycerol lipase	6.00E-20
Ume22616	12.32	5.18E-13	3.34	3.54E-06	Populus trichocarpa predicted protein, mRNA	AT5G58390.1	peroxidase, putative	1.00E-07
Ume01770	12.21	9.21E-17	5.93	2.19E-12	Prunus cerasus vacuolar acid invertase (AI-2) mRNA, complete cds	AT1G62660.1	beta-fructosidase (BFRUCT3)/beta-fructofuranosidase/invertase, vacuolar	5.00E-12
Ume15755	12.09	4.00E-08	5.54	2.11E-09	Lotus japonicus genomic DNA, chromosome 3, clone: LjT46B01, TM0116, complete sequence	AT2G38320.1	unknown protein	5.00E-31
Ume03802	11.79	1.44E-13	6.80	1.83E-11	Populus trichocarpa predicted protein, mRNA	AT4G24890.1	PAP24 (PURPLE ACID PHOSPHATASE 24); acid phosphatase/protein serine/threonine phosphatase	5.00E-07
Ume25683	11.65	3.26E-09	24.23	6.48E-13	PREDICTED: Vitis vinifera hypothetical protein LOC100250551 (LOC100250551), mRNA	AT3G52450.1	PUB22 (PLANT U-BOX 22); ubiquitin-protein ligase	3.00E-24
Ume29703	11.61	6.68E-15	4.46	2.18E-11	Populus trichocarpa predicted protein, mRNA	AT1G78520.1	BEST Arabidopsis thaliana protein match is: glycosyl hydrolase family protein 17 (TAIR:AT2G43670.1)	3.00E-30
Ume23277	11.20	3.13E-15	4.73	1.23E-11	Populus trichocarpa predicted protein, mRNA	AT5G01820.1	ATSR1 (ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 1)	9.00E-06
Ume36796	11.19	1.32E-13	2.16	4.24E-08	Prunus mume PmACO mRNA for 1-aminocyclopropane-1-carboxylate oxidase, partial cds	AT1G12010.1	1-aminocyclopropane-1-carboxylate oxidase, putative/ACC oxidase, putative	2.00E-29
Ume47034	10.88	2.13E-10	5.11	2.91E-10	Glycine max cDNA, clone: GMFL01-17-P07	AT5G64260.1	EXL2 (EXORDIUM LIKE 2)	1.00E-28
Ume51361	10.68	3.25E-13	11.07	7.21E-13	No hits found	AT5G04390.1	zinc finger (C2H2 type) family protein	3.00E-10
Ume57860	10.18	4.70E-14	3.63	4.92E-09	PREDICTED: Vitis vinifera hypothetical protein LOC100246700, transcript variant 3 (LOC100246700), mRNA	AT3G51740.1	IMK2 (INFLORESCENCE MERISTEM RECEPTOR-LIKE KINASE 2)	7.00E-31

Table 3. Genes that were down-regulated in both 0 d to 60 d and 40 d to 60 d cold treatments (more than 10-fold down-regulated in 0 d to 60 d from the top in ascending order).

measurement ID	0 d to 60 d		40 d to 60 d		Target_GB_Description	TAIR9_AGI	TAIR9_Description	TAIR9 *E*-value
	Fold Change	*P*-Value	Fold Change	*P*-value				
Ume26061	0.0016	1.78E-21	0.1168	6.24E-13	Soybean clone JCVI-FLGm-7K15 unknown mRNA	AT4G12510.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.00E-11
Ume58370	0.0042	9.08E-22	0.0714	1.79E-16	Prunus mume Pmdam6 mRNA for dormancy-associated MADS-box transcription factor 6, complete cds	AT2G22540.2	SVP (SHORT VEGETATIVE PHASE); transcription factor/translation repressor, nucleic acid binding	7.00E-06
Ume57227	0.0042	3.81E-21	0.0763	1.04E-15	Prunus mume Pmdam6 mRNA for dormancy-associated MADS-box transcription factor 6, complete cds	AT2G22540.2	SVP (SHORT VEGETATIVE PHASE); transcription factor/translation repressor, nucleic acid binding	8.00E-08
Ume58209	0.0072	4.25E-16	0.0890	9.67E-15	No hits found	AT1G13710.1	CYP78A5; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding	2.00E-10
Ume27998	0.0080	4.65E-14	0.1253	3.42E-11	No hits found	AT1G13710.1	CYP78A5; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding	4.00E-10
Ume08850	0.0083	9.44E-15	0.0391	2.10E-13	Ricinus communis conserved hypothetical protein, mRNA	AT4G10850.1	nodulin MtN3 family protein	1.00E-12
Ume18914	0.0096	3.68E-20	0.1679	5.36E-16	Prunus salicina CI small heat shock protein 2 mRNA, complete cds	AT1G53540.1	17.6 kDa class I small heat shock protein (HSP17.6C-CI) (AA 1-156)	5.00E-53
Ume44725	0.0097	1.04E-17	0.1917	3.12E-12	Prunus persica small heat shock protein mRNA, complete cds	AT1G53540.1	17.6 kDa class I small heat shock protein (HSP17.6C-CI) (AA 1-156)	8.00E-35
Ume15816	0.0098	1.83E-19	0.2504	2.44E-09	Prunus persica dam5 mRNA, complete cds	AT4G24540.1	AGL24 (AGAMOUS-LIKE 24)	1.00E-24
Ume58475	0.0134	2.00E-19	0.1921	1.16E-11	Prunus persica dam5 mRNA, complete cds	AT4G24540.1	AGL24 (AGAMOUS-LIKE 24)	9.00E-08
Ume56241	0.0186	8.02E-20	0.1893	2.00E-12	Ricinus communis cytochrome P450, putative, mRNA	AT3G26295.1	putative cytochrome P450.	9.00E-35
Ume30465	0.0271	4.76E-25	0.4354	7.36E-14	Populus trichocarpa predicted protein, mRNA	AT1G21460.1	nodulin MtN3 family protein	6.00E-31
Ume28753	0.0272	2.58E-16	0.1825	3.09E-11	Populus trichocarpa cytochrome P450 (CYP98A27), mRNA	AT2G40890.1	CYP98A3 (cytochrome P450, family 98, subfamily A, polypeptide 3); monooxygenase/p-coumarate 3-hydroxylase	3.00E-22
Ume21063	0.0299	1.96E-18	0.3342	6.71E-10	PREDICTED: Vitis vinifera hypothetical protein LOC100260796 (LOC100260796), mRNA	AT4G25580.1	stress-responsive protein-related	4.00E-13
Ume18689	0.0305	8.96E-16	0.4641	1.37E-06	Populus trichocarpa predicted protein, mRNA	AT2G33205.1	BEST Arabidopsis thaliana protein match is: MEE55 (maternal effect embryo arrest 55) (TAIR:AT4G13345.1)	9.00E-18
Ume39198	0.0308	2.12E-17	0.4733	1.77E-08	Prunus persica small heat shock protein mRNA, complete cds	AT2G29500.1	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	9.00E-15
Ume29039	0.0359	4.00E-10	0.2439	7.87E-04	Quercus suber CYP86A32 fatty acid omega-hydroxylase mRNA, complete cds	AT5G58860.1	CYP86A1 (CYTOCHROME P450 86 A1); fatty acid (omega-1)-hydroxylase/oxygen binding	1.00E-61
Ume58093	0.0360	6.84E-09	0.3677	1.79E-02	Populus trichocarpa predicted protein, mRNA	AT2G23540.1	GDSL-motif lipase/hydrolase family protein	7.00E-57
Ume35711	0.0362	6.18E-09	0.2368	1.94E-04	No hits found	AT2G40890.1	CYP98A3 (cytochrome P450, family 98, subfamily A, polypeptide 3); monooxygenase/p-coumarate 3-hydroxylase	2.00E-09
Ume49788	0.0377	2.71E-10	0.2500	2.27E-03	PREDICTED: Vitis vinifera hypothetical protein LOC100253445 (LOC100253445), mRNA	AT3G53510.1	ABC transporter family protein	8.00E-22
Ume41078	0.0409	1.67E-10	0.3730	1.21E-02	Populus trichocarpa predicted protein, mRNA	AT4G20390.1	integral membrane family protein	2.00E-26
Ume19059	0.0411	2.27E-10	0.2393	3.67E-04	PREDICTED: Vitis vinifera hypothetical protein LOC100248403 (LOC100248403), mRNA	AT3G53510.1	ABC transporter family protein	9.00E-90
Ume38181	0.0432	7.60E-22	0.4047	1.99E-11	Ricinus communis H(\+)-transporting atpase plant/fungi plasma membrane type, putative, mRNA	AT5G62670.1	AHA11 (Arabidopsis H(+)-ATPase 11); ATPase	5.00E-49
Ume38699	0.0458	1.12E-21	0.4136	1.75E-12	Ricinus communis H(\+)-transporting atpase plant/fungi plasma membrane type, putative, mRNA	AT5G62670.1	AHA11 (Arabidopsis H(+)-ATPase 11); ATPase	1.00E-30
Ume07634	0.0462	9.92E-11	0.2875	1.35E-03	No hits found	AT1G29000.1	heavy-metal-associated domain-containing protein	2.00E-08
Ume29459	0.0464	3.14E-19	0.3019	6.39E-15	Malus x domestica granule bound starch synthase Ia precursor (GBSSIa) mRNA, complete cds	AT1G32900.1	starch synthase, putative	9.00E-17
Ume56039	0.0466	4.17E-18	0.2969	4.45E-14	Malus x domestica granule bound starch synthase Ia precursor (GBSSIa) mRNA, complete cds	AT1G32900.1	starch synthase, putative	3.00E-77
Ume16048	0.0476	6.80E-21	0.2919	1.40E-12	Glycine max mRNA for cysteine proteinase, complete cds	AT1G62710.1	BETA-VPE (BETA VACUOLAR PROCESSING ENZYME); cysteine-type endopeptidase	6.00E-18
Ume45111	0.0498	5.47E-10	0.3313	4.15E-03	Populus trichocarpa predicted protein, mRNA	AT1G68850.1	peroxidase, putative	1.00E-26
Ume33982	0.0498	2.51E-12	0.3005	2.73E-04	Quercus suber CYP86A32 fatty acid omega-hydroxylase mRNA, complete cds	AT5G58860.1	CYP86A1 (CYTOCHROME P450 86 A1); fatty acid (omega-1)-hydroxylase/oxygen binding	8.00E-23
Ume54057	0.0518	2.00E-16	0.3243	2.86E-11	Populus trichocarpa predicted protein, mRNA	AT1G62710.1	BETA-VPE (BETA VACUOLAR PROCESSING ENZYME); cysteine-type endopeptidase	8.00E-18
Ume26620	0.0537	2.67E-14	0.3007	1.24E-05	Prunus persica dam1 mRNA, complete cds	AT4G24540.1	AGL24 (AGAMOUS-LIKE 24)	1.00E-31
Ume38445	0.0540	4.82E-12	0.4563	1.44E-02	Populus trichocarpa predicted protein, mRNA	AT4G20390.1	integral membrane family protein	3.00E-08
Ume52124	0.0562	1.61E-14	0.4318	3.32E-03	Populus trichocarpa predicted protein, mRNA	AT5G07130.1	LAC13 (laccase 13); laccase	2.00E-17
Ume57235	0.0571	2.27E-18	0.3482	1.61E-07	Prunus persica dam3 mRNA, complete cds	AT2G22540.2	SVP (SHORT VEGETATIVE PHASE); transcription factor/translation repressor, nucleic acid binding	3.00E-14
Ume26537	0.0580	1.59E-09	0.4001	2.38E-02	Populus trichocarpa predicted protein, mRNA	AT1G68850.1	peroxidase, putative	3.00E-57
Ume47137	0.0601	2.18E-14	0.1419	2.99E-11	Ricinus communis conserved hypothetical protein, mRNA	AT1G04560.1	AWPM-19-like membrane family protein	3.00E-33
Ume54628	0.0606	6.11E-12	0.2055	1.56E-06	No hits found	AT4G25470.1	CBF2 (C-REPEAT/DRE BINDING FACTOR 2); DNA binding/transcription activator/transcription factor	5.00E-06
Ume24144	0.0615	1.28E-13	0.1276	5.82E-11	Prunus dulcis clone 035NPL unknown gene	AT1G04560.1	AWPM-19-like membrane family protein	1.00E-93
Ume44511	0.0618	3.48E-14	0.1368	2.70E-11	Vitis vinifera contig VV78X174107.9, whole genome shotgun sequence	AT1G04560.1	AWPM-19-like membrane family protein	2.00E-34
Ume20053	0.0634	4.62E-09	0.3460	1.03E-02	PREDICTED: Vitis vinifera hypothetical protein LOC100248401 (LOC100248401), mRNA	AT1G74460.1	GDSL-motif lipase/hydrolase family protein	8.00E-38
Ume27870	0.0642	2.56E-24	0.2803	3.53E-13	No hits found	AT3G03341.1	unknown protein	2.00E-25
Ume35155	0.0658	1.08E-21	0.4655	2.28E-11	Tilia platyphyllos glutaredoxin (Cl31) mRNA, complete cds	AT5G40370.2	glutaredoxin, putative	7.00E-09
Ume48127	0.0698	7.39E-17	0.2531	4.52E-12	No hits found	AT3G03341.1	unknown protein	1.00E-06
Ume56741	0.0702	2.80E-08	0.4035	3.75E-02	PREDICTED: Vitis vinifera hypothetical protein LOC100248401 (LOC100248401), mRNA	AT1G74460.1	GDSL-motif lipase/hydrolase family protein	5.00E-31
Ume57797	0.0711	9.60E-18	0.2646	3.27E-12	No hits found	AT3G03341.1	unknown protein	6.00E-10
Ume26144	0.0730	2.97E-17	0.1762	7.73E-15	PREDICTED: Vitis vinifera hypothetical protein LOC100257513, transcript variant 2 (LOC100257513), mRNA	AT5G46890.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3.00E-15
Ume24187	0.0740	6.28E-17	0.4202	3.94E-07	Ricinus communis conserved hypothetical protein, mRNA	AT5G63350.1	unknown protein	1.00E-15
Ume45437	0.0741	7.44E-09	0.4175	3.04E-02	PREDICTED: Vitis vinifera hypothetical protein LOC100248401 (LOC100248401), mRNA	AT1G74460.1	GDSL-motif lipase/hydrolase family protein	9.00E-08
Ume50984	0.0850	2.67E-24	0.4129	1.52E-08	No hits found	AT1G47610.1	transducin family protein/WD-40 repeat family protein	3.00E-16
Ume31059	0.0868	1.13E-15	0.1971	7.56E-13	No hits found	AT5G46890.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.00E-09
Ume58420	0.0882	4.85E-16	0.2915	1.30E-06	Prunus dulcis clone Pdbcs-L34 putative GAST-like gibberellin regulated protein mRNA, partial cds	AT1G75750.1	GASA1 (GAST1 PROTEIN HOMOLOG 1)	6.00E-17
Ume57732	0.0905	2.36E-15	0.2799	1.28E-06	Prunus dulcis clone Pdbcs-L34 putative GAST-like gibberellin regulated protein mRNA, partial cds	AT1G75750.1	GASA1 (GAST1 PROTEIN HOMOLOG 1)	2.00E-12
Ume39633	0.0920	3.79E-16	0.2787	3.63E-07	Prunus dulcis clone Pdbcs-L34 putative GAST-like gibberellin regulated protein mRNA, partial cds	AT1G75750.1	GASA1 (GAST1 PROTEIN HOMOLOG 1)	4.00E-19
Ume52917	0.0926	1.58E-13	0.4996	7.28E-06	Ricinus communis cinnamoyl-CoA reductase, putative, mRNA	AT2G23910.1	cinnamoyl-CoA reductase-related	2.00E-10
Ume53523	0.0963	6.79E-15	0.2785	5.06E-07	Prunus dulcis clone Pdbcs-L34 putative GAST-like gibberellin regulated protein mRNA, partial cds	AT1G75750.1	GASA1 (GAST1 PROTEIN HOMOLOG 1)	6.00E-16
Ume54090	0.0967	5.58E-15	0.2705	1.03E-06	Prunus dulcis clone Pdbcs-L34 putative GAST-like gibberellin regulated protein mRNA, partial cds	AT1G75750.1	GASA1 (GAST1 PROTEIN HOMOLOG 1)	7.00E-18
Ume40255	0.0995	3.92E-12	0.4618	6.11E-04	Populus trichocarpa predicted protein, mRNA	AT2G23540.1	GDSL-motif lipase/hydrolase family protein	2.00E-31
Ume20777	0.0995	1.85E-11	0.1020	4.80E-11	Ricinus communis conserved hypothetical protein, mRNA	AT5G24470.1	APRR5 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5); transcription regulator/two-component response regulator	3.00E-46

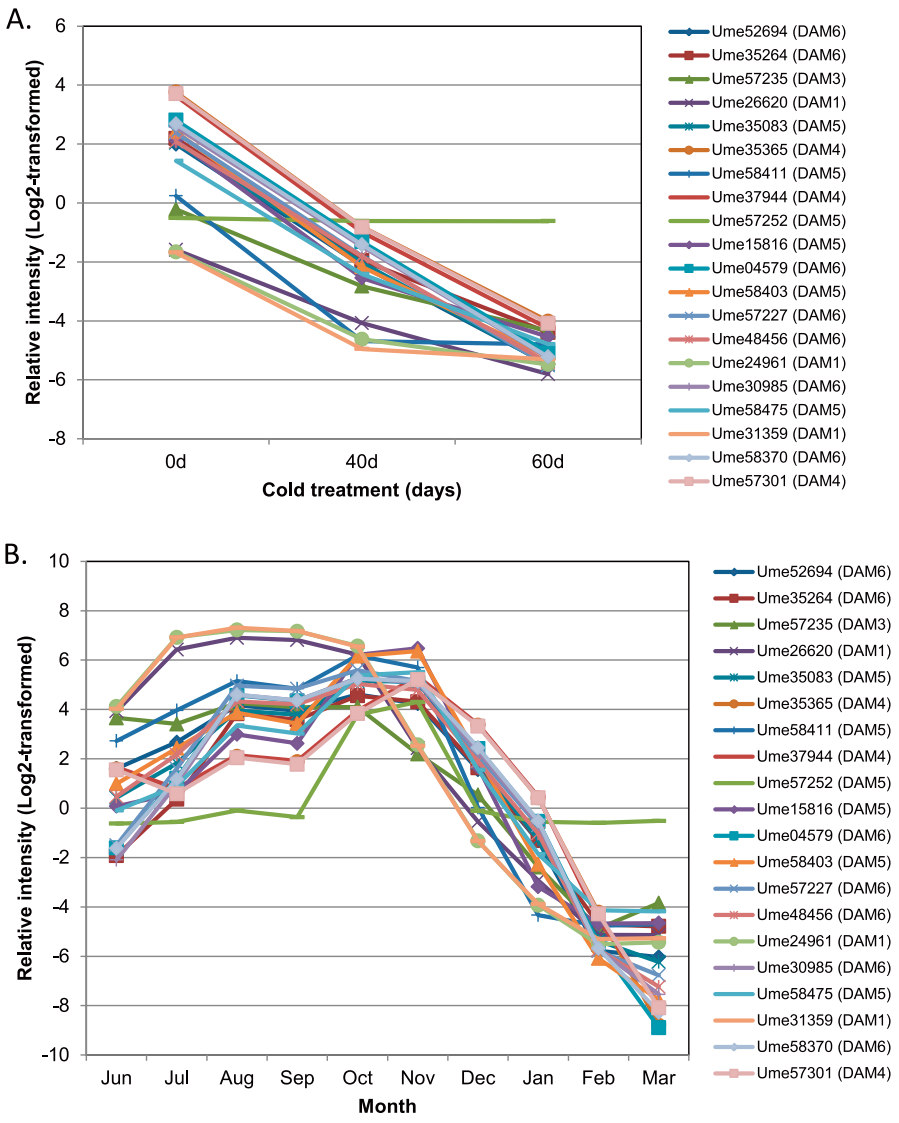

Fig. 2. Microarray results of the relative expression levels of genes annotated as *DAM*s. (A) dataset 1 (chilling treatment) (B) dataset 2 (seasonal change). In legends, annotated gene name for each probe was indicated.

most genes in category (d) were up-regulated in the group 1 months and down-regulated in group 3 months.

Functional categorization of the DEGs

Among the 58627 probes loaded onto the microarray, 26008 probes were annotated in the TAIR9 database (E-value cutoff = $1.0 \times e^{-5}$) and used for PAGE, which is a gene set enrichment analysis that takes expression intensities into account. Forty-seven GO terms categorized as biological processes (The Gene Ontology Consortium, 2000; Table 4) were significantly over-represented ($P < 0.05$) in genes up-regulated by 60 d chilling treatment when compared with 0 and 40 d chilling treatment. According to the GO terms selected, genes related to jasmonic acid biosynthetic and metabolic processes, oxylipin biosynthetic and metabolic processes, and cell-wall modification were significantly up-regulated (Table 4). Twenty GO terms were selected as being significantly over represented ($P < 0.05$) in genes down-regulated by 60 d chilling treatment when compared with 0 and 40 d of chilling treatment (Table 4). Most GO terms selected were related to reproduction and flowering processes. In addition, GO terms of photoperiodism and circadian rhythm/rhythmic processes were also selected.

Verification of microarray analysis by qRT-PCR

As previously described, the two probes with the highest up-regulation were homologues of *Arabidopsis* LOX genes; these genes were assigned to the GO process of the JA biosynthetic process (Table 5). Among the probes assigned to oxylipin/JA biosynthetic/metabolic processes, three probes, Ume31635, Ume13003, and Ume55810, were detected as genes showing marked up-regulation by chilling treatment (Table 5, Fig. 4A). Ume31635 matched to *Arabidopsis LOX2*, whereas Ume 13003 and 55810 matched to *LOX4*; both LOX2

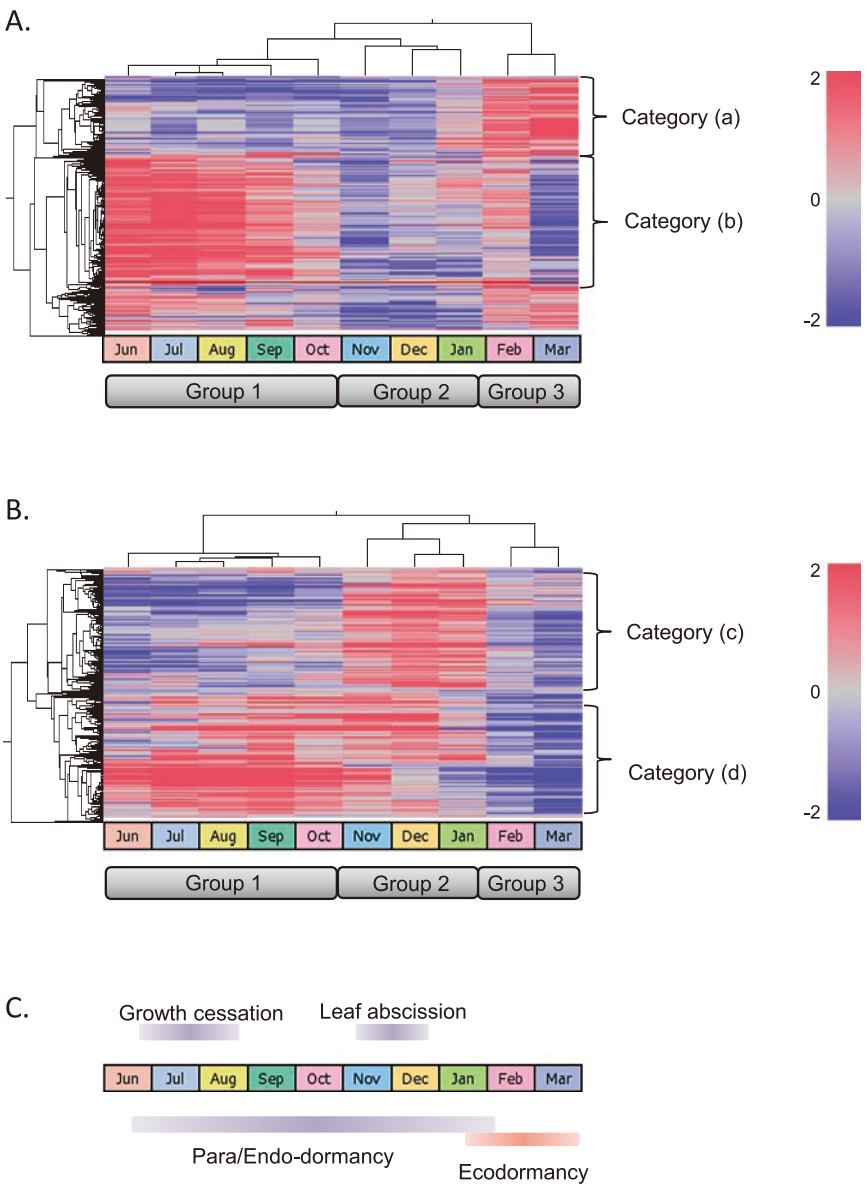

Fig. 3. Cluster analysis of up-regulated (A) and down-regulated (B) genes in Japanese apricot dormant buds by prolonged chilling. Monthly buds were divided into three groups. The genes representing characteristic seasonal expression patterns were divided into categories (a) to (d), (a) down-regulated in groups 1 and 2 months, and up-regulated in group 3 months, (b) up-regulated in group 1 months, and down-regulated in group 2 months and March, (c) down-regulated in groups 1 and 3 months, and up-regulated in group 2 months, (d) up-regulated in groups 1 and 2 months, and down-regulated in group 3 months. (C) Seasonal dormancy status of axillary/lateral buds (Sasaki et al., 2011), and the season when dormancy-related phenological characters are observed in Japanese apricot 'Nanko' in Kyoto, namely growth cessation and defoliation, are indicated.

and LOX4 are involved in polyunsaturated fatty acid catabolism and oxylipin/JA biosynthesis in *Arabidopsis*. According to the microarray results, these genes were slightly down-regulated from June to December, substantially up-regulated in January, and expressed at higher levels in February and March (Fig. 4B). To confirm the microarray data and further characterize gene expression, Japanese apricot unigenes corresponding to Ume31635 and Ume13003/Ume55810 (putative orthologs of peach genes, ppa001631m and ppa001112m, respectively) were selected and subjected to qRT-PCR. As shown in Figure 4C, *PmLOX2*, the ortholog of ppa001631m, was substantially up-regulated by chilling accumulation, whereas *PmLOX4*, the ortholog of ppa001112m, was slightly up-regulated by 60 d chilling treatment relative to 0 d and 40 d chilling treatments.

We also selected genes assigned to the GO terms of circadian rhythm/rhythmic process for qRT-PCR analyses to further verify the microarray results (Table 5). These GOs were chosen because they were identified as being significantly over-represented in the down-regulated gene list by our PAGE analysis. Eleven probes assigned to circadian rhythm/rhythmic process were selected (Table 5). They showed constant expression at 0 d and 40 d chilling treatment, and were down-regulated after 60 d chilling treatment (Fig. 5A). They were up-regulated from

Table 4. Gene ontology terms (biological process) selected by parametric analysis of gene set enrichment (PAGE) in dataset 1 (0, 40, 60 d cold treatment).

GO term	No. of IDs	Description	0 d to 60 d		40 d to 60 d	
			Z-score	*P*-value	Z-score	*P*-value
Up-regulated by 60 d of cold treatment compared to 0 and 40 d : top 20 (highest Z-score) GO terms out of 47 total GO terms (P < 0.05)						
GO:0010200	52	response to chitin	8.1	4.40E-16	7.9	2.20E-15
GO:0009695	19	jasmonic acid biosynthetic process	7.5	7.70E-14	5.6	2.50E-08
GO:0031408	21	oxylipin biosynthetic process	7.4	1.50E-13	5.6	2.20E-08
GO:0009743	102	response to carbohydrate stimulus	6.5	9.10E-11	5.2	2.40E-07
GO:0009694	21	jasmonic acid metabolic process	6.3	4.00E-10	4.8	2.00E-06
GO:0009611	81	response to wounding	6.3	3.20E-10	7.1	1.40E-12
GO:0031407	23	oxylipin metabolic process	6.2	4.40E-10	4.8	1.40E-06
GO:0071554	86	cell wall organization or biogenesis	5.0	5.20E-07	5.5	3.10E-08
GO:0051707	231	response to other organism	4.9	1.00E-06	4.5	6.70E-06
GO:0009664	21	plant-type cell wall organization	4.9	8.00E-07	3.4	7.40E-04
GO:0071555	44	cell wall organization or biogenesis	4.7	2.80E-06	4.3	2.00E-05
GO:0009607	243	response to biotic stimulus	4.6	5.20E-06	4.5	6.80E-06
GO:0009605	179	response to external stimulus	4.5	6.60E-06	3.9	9.70E-05
GO:0006952	190	defense response	4.5	8.20E-06	4.1	4.30E-05
GO:0042545	13	plant-type cell wall modification	4.5	8.10E-06	2.9	3.30E-03
GO:0009620	59	response to fungus	4.4	9.10E-06	5.0	7.20E-07
GO:0051704	295	multi-organism process	4.2	2.60E-05	3.9	8.20E-05
GO:0007166	88	cell surface receptor linked signaling pathway	4.0	7.80E-05	2.4	1.90E-02
GO:0071669	50	plant-type cell wall organization or biogenesis	3.7	1.90E-04	5.0	6.90E-07
GO:0044281	814	small molecule metabolic process	3.4	7.20E-04	9.2	0.00E + 00
Down-regulated by 60 d of cold treatment compared to 0 and 40 d : total 20 GO terms (P < 0.05)						
GO:0010228	43	vegetative to reproductive phase transition of meristem	–3.9	8.20E-05	–4.2	2.70E-05
GO:0048573	23	photoperiodism, flowering	–3.6	3.40E-04	–3.8	1.30E-04
GO:0009648	28	photoperiodism	–3.4	5.70E-04	–3.9	9.40E-05
GO:0042538	25	hyperosmotic salinity response	–3.0	3.00E-03	–2.3	2.20E-02
GO:0048511	30	rhythmic process	–3.0	3.70E-02	–5.7	1.00E-08
GO:0007623	30	circadian rhythm	–3.0	3.70E-02	–5.7	1.00E-08
GO:0003006	431	reproductive developmental process	–2.9	4.60E-02	–5.2	2.00E-07
GO:0000003	499	reproduction	–2.9	4.30E-03	–4.4	1.00E-05
GO:0048608	431	reproductive structure development	–2.9	4.60E-02	–5.2	5.80E-06
GO:0051094	25	positive regulation of developmental process	–2.9	3.30E-03	–2.0	4.90E-02
GO:0006972	28	hyperosmotic response	–2.9	3.60E-03	–2.0	4.60E-02
GO:0009791	507	post-embryonic development	–2.9	3.60E-03	–5.7	1.10E-08
GO:0009908	176	flower development	–2.8	5.10E-03	–5.0	5.00E-07
GO:0022414	492	reproductive process	–2.8	5.30E-03	–4.6	4.80E-06
GO:0048582	24	positive regulation of post-embryonic development	–2.7	6.40E-03	–2.0	4.30E-02
GO:0048518	110	positive regulation of biological process	–2.6	9.30E-03	–3.1	2.00E-03
GO:0006396	146	RNA processing	–2.3	2.30E-02	–3.3	1.10E-03
GO:0048580	86	regulation of post-embryonic development	–2.2	2.50E-02	–2.9	3.80E-03
GO:0071478	22	cellular response to radiation	–2.0	4.90E-02	–3.1	1.70E-03
GO:0071482	22	cellular response to light stimulus	–2.0	4.70E-02	–3.1	1.70E-03

June to July, down-regulated from summer to autumn, slightly up-regulated in winter, and down-regulated from January to March (Fig. 5B). Japanese apricot unigenes corresponding to Ume20777 and Ume17499 (putative orthologs of peach genes, ppa002188m and ppa002863m, respectively) were selected and subjected to qRT-PCR. ppa002188m and ppa002863m were orthologs of *Arabidopsis PSEUDO RESPOSE REGULATOR5* (*PRR5*) and *FLAVIN-BINDING KELCH REPEAT F-BOX* (*FKF1*), respectively, and both of these *Arabidopsis* genes were related to circadian clock controlled genes (Table 5). As shown in Figure 5C, both *PmPRR5* and *PmFKF1*, the ortholog of ppa002188m and ppa002863m, respectively, were down-regulated by 60 d chilling treatment relative to the 0 d and 40 d chilling treatments.

Table 5. The properties of probes belonging to the selected GO terms analyzed by PAGE (see Figs. 4 and 5).

measurement ID	Fold Change (0 d to 60 d)	*P*-Value	Annotation using Arabidopsis thaliana (TAIR9) database		
			TAIR9_Description	Gene ID	*E*-value
1. GO terms: oxylipin/jasmonic acid biosynthetic/metabolic process					
Ume31635	604.52	5.16E-14	LOX2 (LIPOXYGENASE 2)	AT3G45140.1	6.00E-17
Ume13003	118.65	2.65E-19	lipoxygenase, putative	AT1G72520.1	5.00E-11
Ume55810	64.61	4.69E-13	lipoxygenase, putative	AT1G72520.1	0.03
Ume01721	4.50	2.36E-17	DAD1 (DEFECTIVE ANTHER DEHISCENCE 1)	AT2G44810.1	0.002
Ume50019	4.21	2.26E-16	DAD1 (DEFECTIVE ANTHER DEHISCENCE 1)	AT2G44810.1	6.00E-06
Ume24976	3.86	2.17E-11	LOX3	AT1G17420.1	1.00E-87
Ume23157	3.64	2.20E-09	lipoxygenase, putative	AT1G72520.1	4.00E-53
Ume16784	3.27	8.99E-07	AOC4 (ALLENE OXIDE CYCLASE 4)	AT1G13280.1	9.00E-32
Ume30264	3.22	2.54E-10	LOX3	AT1G17420.1	2.00E-50
Ume33131	3.19	9.64E-10	LOX3	AT1G17420.1	2.00E-24
Ume33113	3.10	1.93E-09	lipoxygenase, putative	AT1G72520.1	2.00E-08
Ume14112	3.08	4.95E-09	lipoxygenase, putative	AT1G17420.1	3.00E-14
Ume30834	3.07	1.14E-09	LOX3	AT1G17420.1	9.00E-35
Ume46289	3.02	7.35E-09	LOX3	AT1G17420.1	2.00E-36
Ume33118	2.92	1.97E-08	LOX3	AT1G17420.1	3.00E-12
Ume48452	2.85	2.26E-14	LOX3	AT1G17420.1	4.00E-13
Ume40396	2.57	1.24E-10	lipoxygenase, putative	AT1G72520.1	0.007
Ume07899	2.53	6.38E-10	lipoxygenase, putative	AT1G72520.1	8.00E-04
Ume18381	2.44	1.00E-08	LOX3	AT1G17420.1	5.00E-39
Ume46544	2.32	2.48E-09	LOX3	AT1G17420.1	1.00E-27
Ume44226	2.19	2.58E-09	LOX3	AT1G17420.1	1.00E-26
Ume10626	2.15	1.27E-08	LOX3	AT1G17420.1	8.00E-05
2. GO terms: circadian rhythm and rhythmic process					
Ume20777	0.09947	1.85E-11	APRR5 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5)	AT5G24470.1	3.00E-46
Ume17499	0.11289	7.23E-20	FKF1 (FLAVIN-BINDING, KELCH REPEAT, F BOX 1)	AT1G68050.1	3.00E-69
Ume58335	0.11950	3.05E-19	APRR5 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5)	AT5G24470.1	5.00E-14
Ume49834	0.14614	6.12E-14	FKF1 (FLAVIN-BINDING, KELCH REPEAT, F BOX 1)	AT1G68050.1	1.00E-17
Ume21890	0.15323	2.50E-16	APRR5 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5)	AT5G24470.1	4.00E-27
Ume50310	0.15476	1.80E-10	FKF1 (FLAVIN-BINDING, KELCH REPEAT, F BOX 1)	AT1G68050.1	3.00E-33
Ume42634	0.31887	9.67E-10	CCR2 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2)	AT2G21660.2	2.00E-11
Ume23393	0.43132	7.81E-13	PRR7 (PSEUDO-RESPONSE REGULATOR 7)	AT5G02810.1	1.00E-21
Ume42235	0.43332	4.75E-15	PRR7 (PSEUDO-RESPONSE REGULATOR 7)	AT5G02810.1	8.00E-22
Ume38487	0.44717	2.33E-12	PRR7 (PSEUDO-RESPONSE REGULATOR 7)	AT5G02810.1	4.00E-16
Ume23054	0.46861	1.59E-10	APRR9 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 9)	AT2G46790.1	5.00E-13

Discussion

We constructed a Japanese apricot custom oligoarray (60 mer) containing 58627 independent bud ESTs covering genes involved in numerous kinds of biological processes. Although comprehensive transcriptomic analysis techniques such as pyrosequencing (Habu et al., 2012) and Illumina HiSeq sequencing (Shi et al., 2012) have previously been employed in the study of Japanese apricot, to our knowledge, this is the first report of the use of microarray technology for comprehensive gene expression analysis of Japanese apricot. The probes derived from Japanese apricot dormant bud ESTs matched approximately 60% of all predicted peach mRNAs (data not shown). Considering that the ratios of expressed genes range from 40 to 80% of all predicted genes in *Arabidopsis* and rice (Ma et al., 2005), it is readily expected that our microarray covered a high proportion of genes expressed wholly in Japanese apricot dormant buds.

Yamane et al. (2008) and Sasaki et al. (2011) reported that six *PmDAM*s, *PmDAM1–PmDAM6*, were highly expressed in endodormant Japanese apricot vegetative buds, and that their expression decreased during endodormancy release. All *PmDAM*s were down-regulated by prolonged chilling exposure. To date, the expression patterns of these genes have been more correlated with endodormancy than any other Japanese apricot genes. We analyzed the expression changes of the probes annotated as *DAM*s. Microarray analysis demonstrated that all 20 *DAM* annotated probes except for one probe, Ume57252, were down-regulated by chilling exposure (dataset 1;

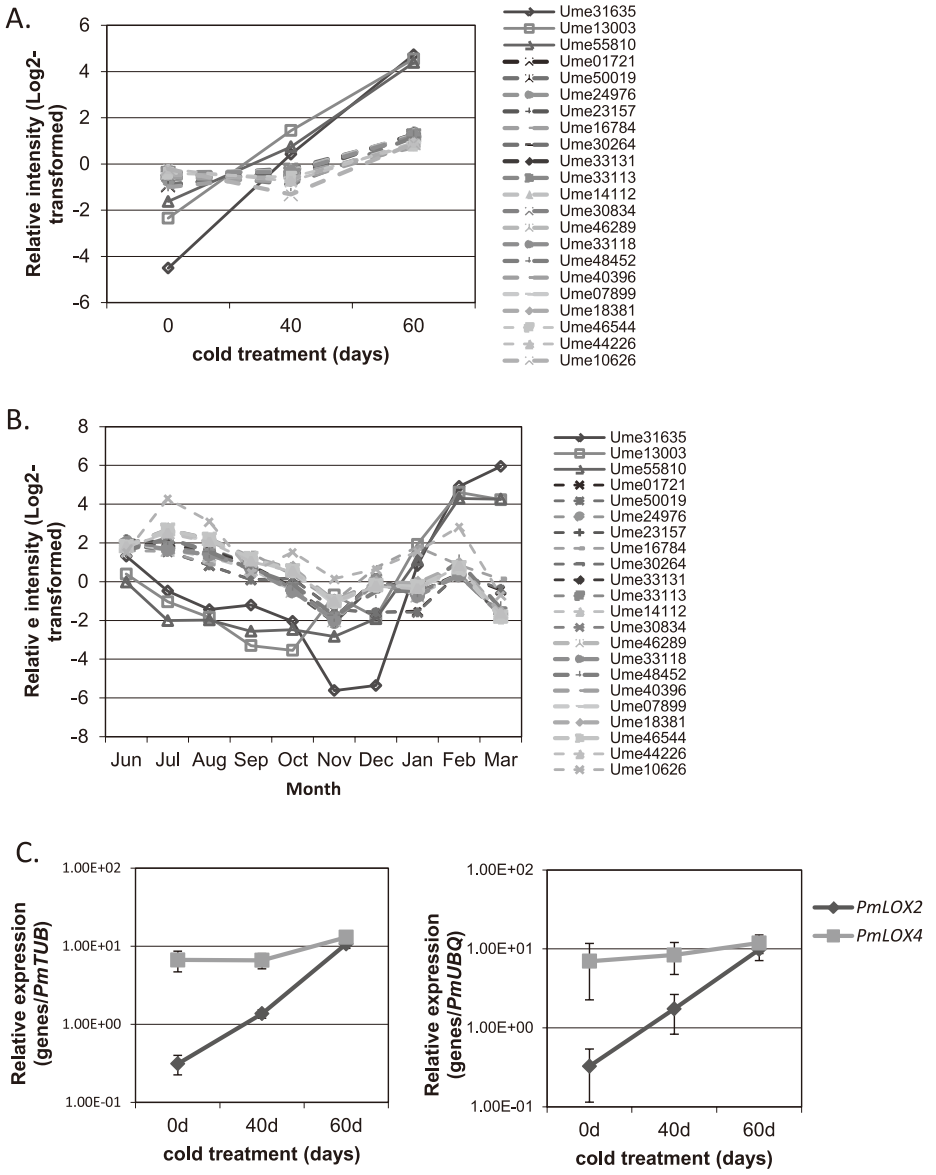

Fig. 4. Expression analysis of selected genes classified within GO terms of oxylipin/jasmonic acid biosynthesis and metabolic process. A: Microarray dataset 1 (chilling treatment). B: Microarray dataset 2 (seasonal change). In A and B, three probes for further characterization (Ume 31635, 13003, and 55810; see text) are shown by bold lines whereas the other probes are shown by dotted lines. Properties of each probe are described in Table 5. C: qRT-PCR analysis of two up-regulated candidate genes (*PmLOX2* and *PmLOX4*) during chilling exposure. The mRNA expression levels are normalized against those of either *PmTUB* (left) or *PmUBQ* (right). Mean and SE (n = 3) are derived from three biological replicates.

Fig. 2A). They were generally up-regulated during the autumn and down-regulated during January when vegetative buds were released from endodormancy (dataset 2; Fig. 2B). These results very much coincided with the previously reported qRT-PCR results (Sasaki et al., 2011) and EST analysis (Habu et al., 2012) of *PmDAM*s, suggesting that our constructed microarray is reliable for monitoring the comprehensive expression changes of bud-expressed genes. In addition to bud dormancy, the microarray constructed in this study will also be applicable for molecular studies aimed at understanding other agronomically important bud physiologies, such as bud phenology, cold hardiness, blooming, and bud abortion.

Seasonal expression analysis of 2345 up-regulated and 1059 down-regulated genes by prolonged chilling treatment suggested the seasonally controlled expression patterns of these genes. Grouping patterns of months by cluster analysis were discussed in association with seasonal transitions of dormancy status and phenological events related to dormancy (Fig. 3C; Sasaki et al., 2011). Chilling-induced genes were down-regulated during the late endodormant season (November–January) and up-regulated in February; some of these genes remained up-regulated in March, but others became down-regulated. Chilling-repressed genes were up-regulated during the late endodormant season, and constantly down-regulated in the ecodormant season (February–March). These results suggest that genes show-

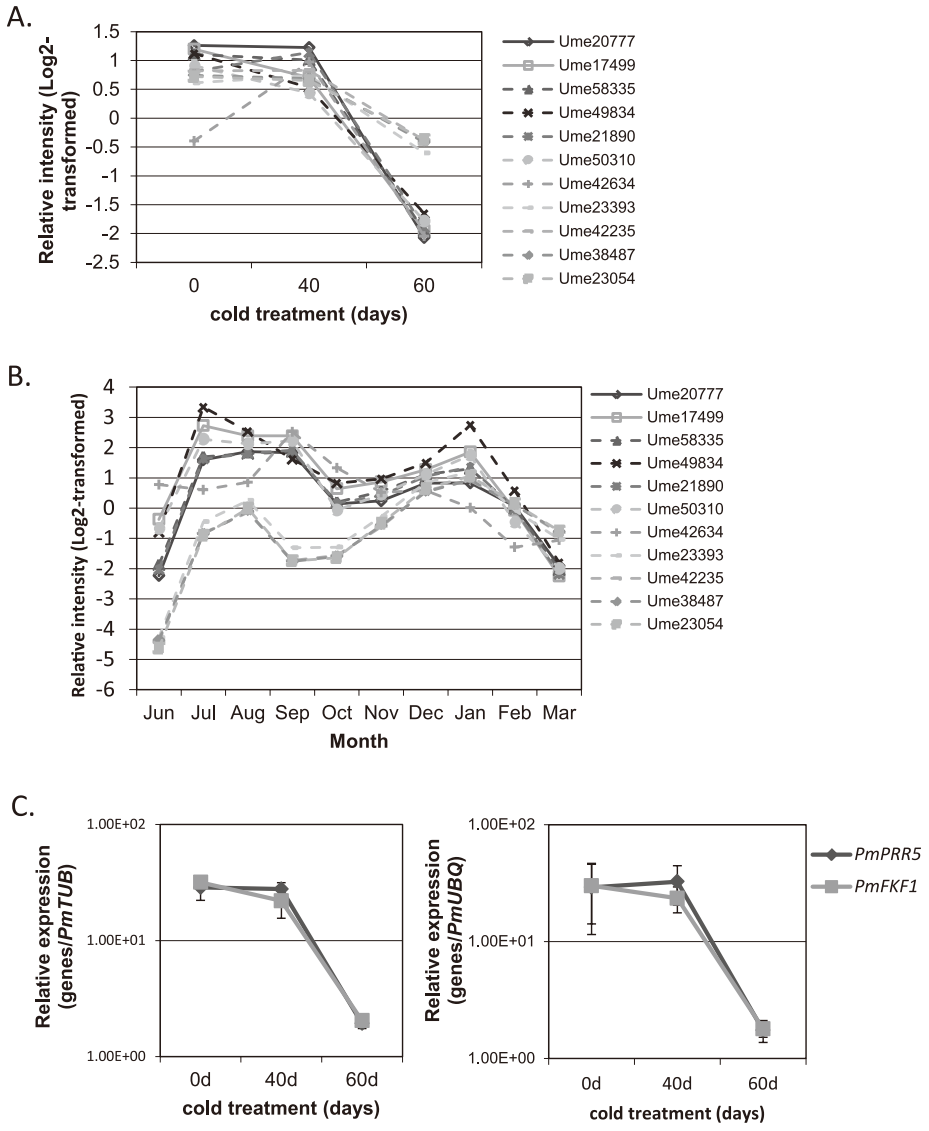

Fig. 5. Expression analysis of selected genes classified within GO terms of circadian rhythm and rhythmic process. A: Microarray dataset 1 (chilling treatment). B: Microarray dataset 2 (seasonal change). In A and B, two probes for further characterization (Ume 20777 and 17499; see text) are shown by bold lines whereas the other probes are shown by dotted lines. Properties of each probe are described in Table 5. C: qRT-PCR analysis of two genes (*PmPRR5* and *PmFKF1*) during chilling exposure. The mRNA expression levels are normalized against those of either *PmTUB* (left) or *PmUBQ* (right). Mean and SE (n = 3) are derived from three biological replicates.

ing prolonged chilling-mediated expression changes also exhibited seasonally coordinated expression from November to February; this coincides with bud phase transition from endodormancy to ecodormancy in Japanese apricot. The dynamic changes of the molecular network among the genes in category (a) and (d) (Fig. 3), which showed ecodormant bud-specific up-regulated and down-regulated expressions, deserve to be further characterized in order to understand the phase transition from endodormancy to ecodormancy. It will be necessary to understand ecodormancy rather than endodormancy to control bud phenology in the field in Japanese apricot, whose endodormancy is relatively shorter than other deciduous fruit tree species.

Cluster analysis based on the expression profiles demonstrated that monthly buds are divided into three groups: June to October; November to January; and February to March. Considering that buds from October and November belong to different clades, temperature changes in autumn rather than photoperiod changes appear to influence the expression changes of the DEGs. One unexpected result was that buds collected in June were included in the same clade as buds in October, when they were in the deep endodormant period. We expected that buds in June were somehow qualitatively rather than quantitatively different from those in October. Nevertheless, the finding that expression patterns of DEGs in June seem to be similar to those in October suggests that buds from June to October were biologically similar at the molecular level. Cooke et al. (2012)

mentioned that the transition from activity to dormancy occurs gradually and takes a long time, and the mechanisms underlying the process of dormancy induction in trees have hardly been dissected. This study raised the possibility that in Japanese apricot the onset of molecular network control to set buds in the endodormant state starts during June, an earlier period than we expected. However, because the seasonal transcriptome analysis in this study did not use biological replicates, and the activity-dormancy cycle can be affected by environmental factors, tree age and vigor, this possibility needs to be verified by repeated year-round studies using substantial biological replicates.

Among the 2345 genes up-regulated by prolonged chilling, the most highly up-regulated gene was *PmLOX2*. qRT-PCR analysis confirmed its up-regulation by chilling treatment. *PmLOX2* was down-regulated during the late endodormant season and up-regulated during January when phase transition of endodormancy to ecodormancy occurs. *Arabidopsis LOX2* encodes a 13-lipoxygenase that converts linolenic acid to 13-hydroperoxy linolenic acid and is involved in polyunsaturated fatty acid catabolism. Reserve mobilization including lipid catabolism could regulate dormancy release and/or the germination of *Arabidopsis* seeds (Finkelstein et al., 2008). On the other hand, a reaction catalyzed by LOXs is a critical step for the jasmonic acid (JA) biosynthesis pathway (Andreou and Feussner, 2009). Our gene set enrichment analysis suggested that oxylipin/JA biosynthetic and metabolic processes were up-regulated by prolonged chilling. However, our qRT-PCR analyses suggested that *PmLOX4* was not highly up-regulated by chilling; this did not support the microarray results. In addition, although some genes involved in JA biosynthesis, such as *DEFECTIVE ANTHER DEHISCENCE1* (*DAD1*) and *ALLENE OXIDE CYCLASE* (*AOC*), were up-regulated by chilling exposure (Table 5), expressions of other genes for JA biosynthetic enzymes, such as 12-oxophytodienoate reductase and allene oxide synthase, were not significantly changed by chilling exposure (data not shown). Thus, further expression studies of genes involved in oxylipin/JA biosynthesis are required in Japanese apricot dormant buds in order to further test the involvement of JA signaling in chilling-induced dormancy transition. So far, no reports have been published that investigated internal JA contents in dormant buds, and the effects of external JA application on bud dormancy breaking of fruit tree species. Recently, Sakamoto et al. (2010) reported that 9-hydroxy-10-oxo-12(Z), 15(Z)-octadecadienoic acid (KODA), one of the oxylipins, was effective for promoting the endodormancy break of flower buds of Japanese pear. Although our preliminary studies suggested no obvious effects of KODA on the dormancy break of Japanese apricot (Yamane et al., In press), it would be interesting to investigate the internal oxylipin/JA contents in dormant buds, and the effects of oxylipins on dormancy breaking of Japanese apricot.

Our PAGE analysis suggested that the GO processes related to the transition from vegetative to reproductive, flowering, and reproductive processes were down-regulated during prolonged chilling treatment. The significant over-representation of these GOs probably reflects the down-regulation of *DAM*s. *DAM*s belong to a class of MADS-box genes, which are known as flowering-related genes in *Arabidopsis* and categorized in these GOs. In addition to these GOs, the GO processes of circadian rhythm and rhythmic process were identified as being down-regulated during prolonged chilling treatment. Both microarray and qRT-PCR analyses demonstrated that prolonged chilling down-regulated Japanese apricot gene homologs of *PRR5* and *FKF1*, which are known to be critical circadian clock and photoperiod-related genes in *Arabidopsis* (reviewed by Song et al., 2010). Some of the genes included within the GO circadian rhythm/rhythmic process classifications were up-regulated from June to July, when axillary buds gradually enter endodormancy, and down-regulated from January to March, during the transition from endodormancy to ecodormancy (Fig. 5B). Similar expression patterns of *PRR5* and *FKF1* have been reported in other plant species. *Populus PRR5* was highly expressed following growth under short-day conditions (Ruttink et al., 2007), and its expression in dormant buds decreased gradually during bud burst (Ibáñez et al., 2010). Enhanced expression of *PRR5* delayed bud burst, suggesting that *PRR5* is a candidate marker gene for the depth of bud dormancy in *Populus* (Cooke et al., 2012). In leafy spurge, *FKF1* was down-regulated in ecodormant buds relative to endodormant buds (Doğramaci et al., 2010). However, microarray analysis of pear dormant buds suggested that *PRR5* was highly expressed in ecodormant buds (February) relative to endodormant buds (November) (Nishitani et al., 2012). Even though this discrepancy suggested that clock gene expression changes differ among plant species, recent findings from *Populus* strongly suggest that the circadian clock-dependent molecular network affects growth cessation and winter dormancy regulation (Cooke et al., 2012; Ibáñez et al., 2010; Ruttink et al., 2007). Clock component genes are known to be involved in temperature sensing in addition to photoperiod sensing. Indeed, clock components play crucial roles in the response to chilling-induced seed dormancy break in *Arabidopsis* (Penfield and Hall, 2009), and chilling-induced dormancy onset in chestnut (*Castanea sativa*) (Ibáñez et al., 2008; Ramos et al., 2005). Thus, further studies focusing on the circadian-clock regulatory network will be required for a fuller understanding of the possible involvement of circadian-clock genes in the dormancy regulation of Japanese apricot and other fruit tree species.

In conclusion, this study provided transcript profiles of Japanese apricot dormant buds in response to prolonged chilling treatments. This work supported previous studies that *PmDAM*s were highly down-regulated by chill-

ing (Habu et al., 2012; Sasaki et al., 2011; Yamane et al., 2008), suggesting their critical roles in dormancy regulation. However, our knowledge about genetic and molecular mechanisms regulating bud dormancy is still very fragmentary. The significantly up-regulated and down-regulated genes identified in this study are good candidates for the control of chilling-mediated physiological events in dormant buds. Further functional characterization of each candidate gene and deciphering the molecular interactions among candidate genes will be required to dissect how chilling temperature regulates bud phenology events, including dormancy and bud break.

Important notes: During the preparation of this paper, an article describing the whole genome sequencing of *P. mume* was published (Zhang et al., 2012). In future, whole genome sequence data incorporated with developing bioinformatics programs will strongly promote omics studies in Japanese apricot and provide us with new insights for understanding agronomically important traits in the species.

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