



RESEARCH PAPER

Transcription profiling of the chilling requirement for bud break in apples: a putative role for *FLC-like* genes

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Abstract

Apple production depends on the fulfilment of a chilling requirement for bud dormancy release. Insufficient winter chilling results in irregular and suboptimal bud break in the spring, with negative impacts on apple yield. Trees from apple cultivars with contrasting chilling requirements for bud break were used to investigate the expression of the entire set of apple genes in response to chilling accumulation in the field and controlled conditions. Total RNA was analysed on the AryANE v.1.0 oligonucleotide microarray chip representing 57 000 apple genes. The data were tested for functional enrichment, and differential expression was confirmed by real-time PCR. The largest number of differentially expressed genes was found in samples treated with cold temperatures. Cold exposure mostly repressed expression of transcripts related to photosynthesis, and long-term cold exposure repressed flavonoid biosynthesis genes. Among the differentially expressed selected candidates, we identified genes whose annotations were related to the circadian clock, hormonal signalling, regulation of growth, and flower development. Two genes, annotated as *FLOWERING LOCUS C-like* and *MADS AFFECTING FLOWERING*, showed strong differential expression in several comparisons. One of these two genes was upregulated in most comparisons involving dormancy release, and this gene's chromosomal position co-localized with the confidence interval of a major quantitative trait locus for the timing of bud break. These results indicate that photosynthesis and auxin transport are major regulatory nodes of apple dormancy and unveil strong candidates for the control of bud dormancy.

Key words: Apple, bud dormancy, Castel Gala, chilling requirement, *FLC-like*, microarray, Royal Gala.

Introduction

Perennial growth in temperate regions demands tolerance to harsh winter conditions. In the autumn, plants growing in such locations may exhibit bud formation and growth cessation

as a mechanism of tolerance to forthcoming winter stresses. This phenomenon, called dormancy, protects sensitive meristematic tissues from damage. Dormancy in buds is classified

Abbreviations: CH, chilling hours; CR, chilling requirement; DE, differentially expressed; EST, expressed sequence tag; GO, Gene Ontology; miRNA, microRNA; QTL, quantitative trait locus; RT-qPCR, reverse transcription quantitative PCR; TCA, tricarboxylic acid.

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as para-, endo-, or ecodormancy when the growth inhibition is driven either by other structures of the same plant, by the dormant bud itself, or by environmental conditions, respectively (Lang *et al.*, 1987). Dormancy is triggered by short photoperiods and/or low temperatures and is released after a certain period of cold exposure, when the bud is left in a quiescent, ecodormant state and is capable of resuming growth when the conditions (temperature, nutrients, and water) are favourable (Cooke *et al.*, 2012; Horvath *et al.*, 2003).

One of the goals of temperate fruit crop breeders is to establish cultivars fully adapted to their growing environment (Denardi *et al.*, 1988; Labuschagné *et al.*, 2002). To have an approximate prediction of phenological behaviour of cultivars during the winter, breeders rely on models of dormancy progression. The classical chilling hour model predicts that each crop species and cultivar requires a certain amount of chilling hours (CH), i.e. hours of temperatures between 0 and 7.2 °C, for endodormancy release (Chandler *et al.*, 1937). This numeric quantity of CH is known as the chilling requirement (CR).

The molecular mechanisms controlling bud dormancy have been the subject of intensive research in the last decade, and many genes are believed to play roles in dormancy establishment and release (Ruttink *et al.*, 2007; Horvath *et al.*, 2008; Gai *et al.*, 2013). In *Populus trichocarpa*, the timing of bud set was largely attributed to the expression levels of *PtFT1*, an orthologue of the *Arabidopsis* florigen *FLOWERING LOCUS T (FT)*. *PtFT1* is induced by the aspen version of *CONSTANS (CO)*, whose expression is affected by day length (Böhlenius *et al.*, 2006). Further investigations established *PtFT1* as a determinant of reproductive onset and its parologue, *PtFT2*, as an inhibitor of growth cessation and bud set (Hsu *et al.*, 2011).

In *Arabidopsis*, *FT* expression, which leads to flowering, is controlled by pathways that respond to autonomous and environmental signals. The acquisition of flowering competence by prolonged cold exposure, also known as vernalization, requires silencing of the flowering repressor *FLOWERING LOCUS C (FLC)* (Amasino and Michaels, 2010). The physiological similarities between vernalization and dormancy release led to the hypothesis that a molecular mechanism analogous to vernalization could be responsible for dormancy release, with the participation of an FLC-like repressor (Horvath *et al.*, 2003; Rohde and Bhalerao, 2007). At the present time, the most promising repressors are the *DORMANCY-ASSOCIATED MADS-box (DAM)* genes. They were discovered by positional cloning after linkage map analysis of the *evergrowing* peach mutant, which is unable to enter dormancy (Bielenberg *et al.*, 2008).

Circadian rhythm and gibberellin response genes were also shown to participate in dormancy cycling in *Populus* trees. Downregulation of *P. trichocarpa LATE ELONGATED HYPOCOTYL (PtLHY)* expression, a circadian clock core component, compromised cold acclimation and delayed bud burst (Ibáñez *et al.*, 2010). Gibberellin application can substitute chilling, and gibberellin-induced expression of β-1,3-glucanases was reported to have an important role in dormancy release (Rinne *et al.*, 2011).

It is believed that almost all major plant hormones play a role in at least one dormancy stage (Horvath, 2009). In potato, cytokinins are essential to break dormancy in buds (Hartmann *et al.*, 2011). However, the role of cytokinins in endodormancy has not been studied extensively (Cooke *et al.*, 2012). The role of abscisic acid is unclear, but it may participate in dormancy-related processes, such as bud formation and cold acclimation (Cooke *et al.*, 2012). Finally, auxins are a major component of paradormancy, and auxin-mediated inhibition of bud break has been demonstrated in apple (Faust *et al.*, 1997).

The CR in apple is highly variable across cultivars, ranging from 200 to 1100 CH (Labuschagné *et al.*, 2002), and it can be considered a genetically determined trait. The Gala Standard cultivar requires approximately 600 CH to break its dormancy, and the same is true for most cultivars derived from it (e.g. 'Royal Gala'). Conversely, the Castel Gala cultivar is a 'Gala Standard' mutation featuring a low CR of approximately 300 CH while retaining all other traits (Anzanello *et al.*, 2014). This provides a unique opportunity for dormancy research because it allows the comparison between two nearly identical genetic backgrounds that, to the best of our knowledge, differ only in the CR.

Genetic segregation studies on CR are complicated by the quantitative nature of bud dormancy and the influence of environmental factors such as temperature, drought, and nutrient availability. Because there is no genetic marker for CR in apple and it is a trait that manifests only after the dormancy cycles commence, breeding efforts for CR adjustment usually take several years. Recently, a major quantitative trait locus (QTL) for the timing of bud break, a trait closely associated with CR, was found to be associated with regions of chromosome 9 (van Dyk *et al.* 2010; Celton *et al.*, 2011). Celton *et al.* (2011) positioned the confidence interval for the QTL in the first 4 Mb of this chromosome (hereafter called LG9-QTL), a result also found by our group in an independent mapping population (Tessele, 2012). However, genomic dissection of the QTL revealed no unique strong candidate gene for the control of timing of bud break. Furthermore, transcriptomic studies comparing apple buds across dormancy stages have not been reported, with the exception of the work by Pichler *et al.* (2007). They analysed transcripts from autumn and winter buds on a 15K oligonucleotide chip designed using sequences from available expressed sequence tag (EST) libraries. Microarray analyses revealed a significant number of genes up- and downregulated during winter. However, no inferences were made from these data from the perspective of functional genomics. One reason for this could be that, at the time of the study, there was insufficient literature on the molecular control of bud dormancy, as well as a relatively low availability of databases for apple and other temperate fruit trees.

Since then, the number of resources for high-throughput functional genomics analyses has grown exponentially, including for apple (Troggio *et al.*, 2012). In 2010, a genome sequence of apple was made publicly available, which greatly accelerated apple genomics research (Velasco *et al.*, 2010). Apple genome data analysis estimated a number of 57K

genes, which was the basis for the design of a full genome microarray chip for apple, the AryANE chip (Celtón *et al.*, 2014). This work aimed to identify differential expression of apple genes across bud dormancy stages. Closed terminal buds exhibiting distinct dormancy status were screened for differential gene expression using the AryANE chip. These results were analysed by singular enrichment analysis, and differential expression for 15 selected transcripts was confirmed by reverse transcription followed by quantitative PCR (RT-qPCR). Cold-treated buds exhibited a great number of differentially expressed (DE) genes when compared with buds in the field. DE genes were enriched in Gene Ontology (GO) terms related to photosynthesis, the citrate cycle, ribosome biogenesis, and flavonoid biosynthesis. RT-qPCR analysis of selected genes corroborated the microarray results, and supported the selection of putative transcription factors located inside the LG9-QTL as candidates for regulating dormancy release.

Materials and methods

Location of orchards

The apple trees used for this study were grown in a commercial orchard located at the city of Papanduva, SC, Brazil ($26^{\circ}26'68''S$, $50^{\circ}05'47''W$, 788 m altitude). In this region, the climate is classified as Cfb according to the Köppen climate classification. March, April, and May (autumn) have historical mean minimum temperatures of $9\text{--}14^{\circ}\text{C}$ and mean maximum temperatures of $18\text{--}25^{\circ}$. June and July (winter) have mean minimum temperatures of $5\text{--}8^{\circ}\text{C}$ and mean maximum temperatures of $18\text{--}20^{\circ}$. The annual rainfall totals 1300–1500 mm and is evenly distributed. Field temperatures were recorded hourly by an automatic weather monitoring station.

Plant material and cold treatments

'Royal Gala' trees, at the time of first harvesting (2009), were 5 years old, grafted on 'Marubakaido' rootstock with 'M9' as the interstock, while 'Castel Gala' trees were 3 years old and grafted on 'M9' rootstock. The difference in the grafting system was shown not to alter the timing of bud phenology (Petri *et al.*, 2012). All trees were in the adult stage and fully productive. 'Royal Gala' and 'Castel Gala' twigs (~20 cm, no more than 10 samples per tree) bearing a dormant, terminal flower bud (A stage according to Fleckinger, 1964) were sampled in March, May, and June 2009 (autumn and winter in the southern hemisphere). Twigs were wrapped in moist paper and shipped to the laboratory, where they were surface sterilized. For the bud break assay, 40 twigs were fixed in floral foam inside a growth chamber under forcing conditions ($25 \pm 1.5^{\circ}\text{C}$, 12 h photoperiod, 70% relative humidity). The percentages of maximum bud break were determined from the total number of terminal buds showing green tips after 35 d of forcing treatment.

For cold treatments, twigs were placed in BOD-type chambers at 6°C in the vertical position. After 1- or 2-week intervals, 40 twigs were forced as described above, and 10–20 terminal buds were frozen in liquid N_2 and stored at -80°C . A schematic representation of the 2009 treatments and harvesting points is depicted in Fig. 1.

Samplings were repeated in April, May, June, and July 2010 to select comparisons from a different growth cycle as biological replicates. Several cold treatments were tested, and the ones that better replicated the results from 2009, with respect to the total CH accumulated (both CH from the field and from controlled conditions) and the change in percentage of maximum bud break, were used. A description of the 2009 and 2010 biological replicates can be found in Supplementary Table S1 at JXB online.

RNA isolation

Total RNA was isolated from samples composed of 10–20 buds as described by Zeng and Yang (2002) and following the modifications of Reid *et al.* (2006). Isolated RNA was further purified using a solid-phase column purification kit (NucleoSpin; Macherey-Nagel, PA, USA). Contaminating DNAs were removed using a TURBO DNA-free kit (Life Technologies, Gaithersburg, MD, USA).

Hybridizations

RNA samples were screened for differential gene expression by hybridization with the AryANE chip (INRA), according to Celtón *et al.* (2014). Approximately 400 ng of total RNA was amplified using an Ambion messageAmp II kit (Ambion, Austin, TX, USA). Amplified RNAs (5 µg) were used for sample labelling by RT in the presence of Cy3- or Cy5-CTP. After purification and quantification, labelled samples were combined as 30 pmol for each dye and co-hybridized to the AryANE $12 \times 135\text{K}$ arrays. After washing and drying, the fluorescence signals were read by a MS200 scanner (NimbleGen, Madison, WI, USA). NimbleScan Software v.2.4 was used to extract pair-data files from the scanned images. For each comparison, two biological replicates, one from each growth

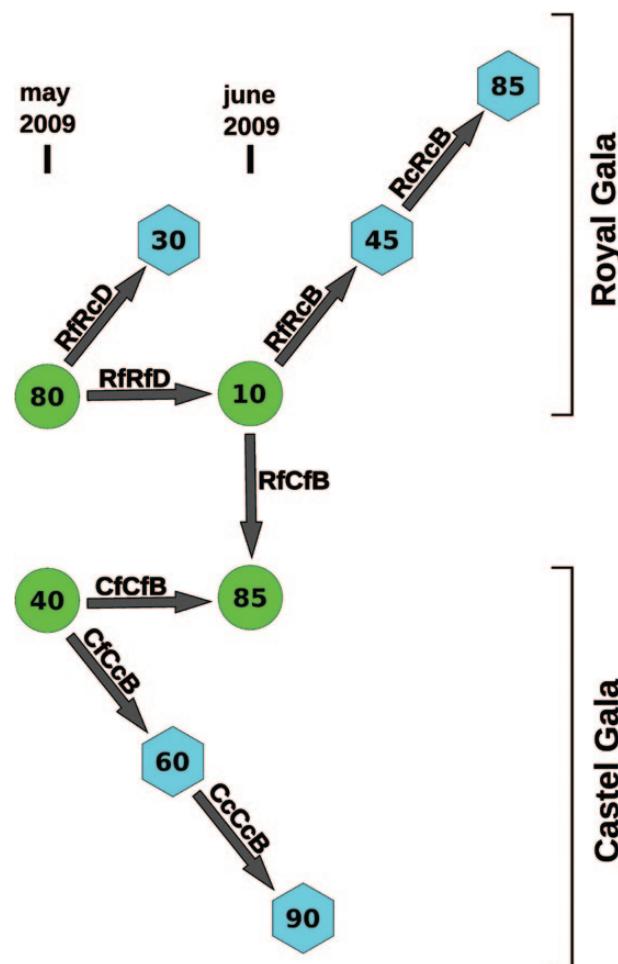


Fig. 1. Experimental design. Samples from closed terminal buds of 'Royal Gala' and 'Castel Gala' apple trees were harvested in May and June 2009 and exposed to 1 or 2 weeks of cold treatments. Circles denote buds frozen in the field, and hexagons represent cold-treated buds. Numbers inside the shapes are the bud break rates for each sample. Arrows demonstrate the pairs of samples compared in the hybridizations. Five-letter codes alongside the arrows are comparison names (see Materials and methods for their designation). (This figure is available in colour at JXB online.)

cycle (2009 and its 2010 counterpart; see [Supplementary Table S1](#)), were analysed in a dye-switch design, as described in [Depuydt et al. \(2009\)](#).

All statistical analyses were performed using the R language ([R Development Core Team, 2014](#)). The data were normalized using the LOWESS method, the differential expression analyses were performed using the lmFit function, and the Bayes moderated *t*-test was performed using the package LIMMA ([Smyth, 2005](#)) from the Bioconductor project. Genes considered to be DE showed transcript level variation greater than 1.5-fold in at least one comparison, for $P < 0.01$. The normalized microarray data were deposited at NCBI's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession code GSE64717.

Comparisons were designed in the most comprehensibly form possible in order to follow transcript accumulation changes during dormancy entrance and release. Because each sample had a particular set of properties (bud break rate, cultivar, etc.), a five-letter code representing sample conditions in each comparison was devised. The code rationale is as follows: the first and second letters are the cultivar (R, 'Royal Gala'; C, 'Castel Gala') and treatment (f, field-harvested; c, cold-treated), respectively, for the first sample. The third and fourth letters indicate the same for the second sample. Finally, the fifth letter shows whether the bud break rate of the second sample was higher (B, bud break) or lower (D, dormancy) than the first sample. This labelling is shown graphically in [Fig. 1](#).

Singular enrichment analysis

DE genes (>1.5-fold change, $P < 0.01$) were tested for enrichment in GO functional classifications using the Blast2GO program ([Götz et al., 2008](#)). GO term annotations were downloaded from the apple genome and validated in the program (i.e. only the most specific terms for each ontology branch were kept). DE genes were also tested for enrichment in KEGG metabolic pathways using the KOBAS software ([Xie et al., 2011](#)).

Validation by real-time PCR

Gene-specific primers for each selected transcript were designed using Primer3 ([Rozen et al. 2000](#)) and checked for stable putative secondary structures with IDT's OligoAnalyzer (<http://www.idtdna.com/analyizer/applications/oligoanalyzer>). From each RNA sample, cDNAs were synthesized using a GeneAmp RNA PCR Core kit (Life Technologies) following the manufacturer's instructions. The amplification reactions were performed in a StepOnePlusTM Real-Time PCR System (Life Technologies) as follows: 10 µl of diluted cDNA (1:100), 200 nM of each primer, 50 µM of each dNTP, 0.25 units of PlatinumTM Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1× Buffer Solution (Invitrogen), 1.5 mM MgCl₂, 1× SYBR GreenTM (Invitrogen), and 1× ROX reference dye (Invitrogen). The reactions were performed in technical quadruplicates for each sample. RT-qPCR assays were conducted with the following cycling program: 95 °C for 10 min for enzyme activation, 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min, and finally a dissociation curve between 60 and 95 °C. Relative expression calculations followed Pfaffl mathematics ([Pfaffl, 2001](#)) for the following three reference genes established previously as being constitutively expressed in apple buds ([Perini et al., 2014](#)): *Accumulation and replication of chloroplast 5 (ARC5, MDP0000138874)*, *Malate dehydrogenase (MDH, MDP0000197620)*, and a WD40 domain-containing protein (*WD40, MDP0000168479*). Primer specificity was checked by dissociation curves, agarose gel electrophoresis, and Sanger sequencing of generated amplicons.

Results

In an attempt to discover DE genes during dormancy in terminal buds of apple, this work explored the changes in steady-state mRNA levels in buds with diverse dormancy status (i.e.

bud break rates). The differences in dormancy were a result of either cold treatments in controlled conditions, the dormancy progression under field conditions, as evaluated by the comparison of samples harvested in different dates, or genotype-specific effects in buds from 'Royal Gala' and 'Castel Gala' trees placed side by side and sampled on the same day. A schematic diagram of the experimental design can be found in [Fig. 1](#).

To obtain a more robust result, bud samples from two different years were used as biological replicates. Because these samples originated from other growth cycles and have had slightly different cold treatments, we relied on CH accumulation and changes in bud break rate as indicators of dormancy status ([Fig. 1](#) and [Supplementary Table S1](#)). In March 2009, for both cultivars, samples failed to respond to cold treatments, and budbreak rates of field-harvested buds were very close to those of the May 2009 samples. Hence, the March 2009 samples were not included in the comparisons.

Over 8000 genes (8193) were DE across the comparisons, most of them identified in cold-treated 'Royal Gala' buds compared with buds frozen in the field ([Table 1](#)). Chilled 'Castel Gala' buds, however, did not show the same number of DE genes when compared with chilled 'Royal Gala' buds. Unexpectedly, in 2010, constant cold temperatures (3 °C) did not significantly alter the competence of 'Castel Gala' terminal buds to break. Another set of buds, exposed to an alternative temperature regime (3 and 15 °C at 12 h intervals) did show an increase in bud break rates. These samples were used as a biological replicate to 2009 'Castel Gala' buds exposed to constant cold. This difference over replicates may account for the lower number of DE genes in CfCcB compared with the 'Royal Gala' assays.

A large number of genes were DE in more than one comparison. A pairwise table of comparisons containing the number of genes up- or downregulated is shown in [Table 2](#). More than 800 genes were downregulated in both comparisons involving chilling of 'Royal Gala' buds. This number fell to approximately 400 for upregulated genes in common for these comparisons ([Table 2](#)).

To test for functional enrichment among DE genes, GO terms and KEGG pathway over-representation were assessed using the Blast2GO and KOBAS tools, respectively ([Götz et al., 2008; Xie et al., 2011](#)). Only coincident term enrichment results for both programs are described. Full reports are available online ([Supplementary Table S2](#) at JXB online).

Table 1. Total number of DE genes across comparisons

Comparison	Induced	Repressed	Total DE
RfRcD	1181	2424	3605
CfCcB	281	161	442
CcCcB	124	374	498
RfRfD	137	332	469
CfCfB	226	282	508
RfCfB	91	77	168
RfRcB	2127	2228	4355
RcRcB	170	287	457

Table 2. Number of coincident DE genes between pairs of comparisons

Below diagonal indicates downregulated genes and above the diagonal indicates upregulated genes.

	RfRcD	CfCcB	CcCcB	RfRfD	CfCfB	RfCfB	RfRcB	RcRcB
RfRcD		73	5	26	11	0	393	42
CfCcB	68		5	11	6	0	105	1
CcCcB	13	3		6	5	2	7	0
RfRfD	26	24	9		26	0	8	8
CfCfB	19	12	19	33		4	10	0
RfCfB	1	0	4	1	2		4	0
RfRcB	810	55	12	33	10	7		9
RcRcB	11	2	22	7	3	2		24

In the RfRcD comparison, both programs detected a downregulation of transcripts related to photosynthesis and an upregulation of transcripts involved in ribosome biogenesis. Photosynthesis terms were also observed more frequently in downregulated transcripts of the RfRcB comparison. The programs also found an over-representation of flavonoid biosynthesis genes among downregulated transcripts in RcRcB and of tricarboxylic acid (TCA) cycle genes in upregulated transcripts of RfRcB. Microarray data for the groups of genes presenting functional terms that were significantly enriched are depicted in Figs 2 and 3.

Two subgroups of photosynthesis-related genes showed differential regulation during dormancy

Hierarchical clustering of photosynthesis-related gene expression results found a group of 11 genes, most of them encoding components of photosystem I and II, with transcriptional induction in RcRcB, CcCcB, and, to a lesser extent, RfRcD (Fig. 2). Promoter sequences (1 kb upstream of the predicted translational start site) for eight of these genes were available in the apple genome database (Velasco *et al.*, 2010), as well as for 166 of the remaining DE photosynthesis-related genes. In an attempt to identify candidate signalling pathways that influence the expression of these genes, promoter sequences were scanned for conserved *cis*-acting elements using the PLACE database (Higo *et al.*, 1999). Of the eight promoters of photosynthesis genes induced at the end of dormancy, only one (12.5%) contained a G-box conserved motif (CACGTG) or a G-box-like sequence (ACGTG). However, of the 166 promoters scanned for the remaining genes, 65 (39.1%) presented at least one G-box motif and 123 (74.0%) contained the G-box-like sequence. No other conserved element presented this contrasting representation between these two promoter sets (data not shown).

Some non-coding transcripts showed differential expression during bud dormancy

The AryANE microarray chip also features oligonucleotides designed for the detection of microRNA (miRNA) genes and antisense transcripts. This work identified a number of conserved miRNA genes as DE, almost all downregulated (Table 3). Their potential targets included AUXIN

RESPONSIVE FACTORS (ARFs), HD-zip transcription factors, and GAMYB-like coding transcripts. A significant number of putative antisense transcripts (1557) was differentially regulated in the samples analysed. Almost half (735, 47.2%) of the predicted apple genes showing this differential antisense expression were also DE as evaluated by sense oligonucleotides (Supplementary Table S3 at JXB online). Among these 735 genes, 345 (46.9%) exhibited the same expression profile between sense and antisense transcripts in all comparisons.

Validation of microarray results highlights participation of flowering time, the circadian clock, and hormone signalling pathways

Gene selection for microarray validation gave priority to predicted genes that fitted in one or more of the following criteria: (i) DE in more than one comparison; (ii) located inside the LG9-QTL; and (iii) functions in dormancy and/or flowering time regulation, according to current models (Horvath *et al.*, 2003; Amasino and Michaels, 2010; Rinne *et al.*, 2011). Confirmation of differential expression was accomplished by RT-qPCR employing the same RNA samples isolated for the hybridizations. Microarray and RT-qPCR results for selected predicted transcripts are shown in Fig. 4. For the sake of clarity, the names of the best matches to *Arabidopsis* and/or NCBI databases are used to refer to the selected transcripts, except for genes already characterized for apple (*AFL1*, *MdFT*, and *MdDHN2*). Genome codes for selected predicted genes, BlastX results, and the primers used are available in Supplementary Table S4 at JXB online.

The expression of two apple genes, *AFL1* and *MdFT1*, previously found to be involved in flowering time (Kotoda *et al.*, 2010), was repressed in RfRcB. Interestingly, *MdFT1* was 2.5-fold induced in 'Royal Gala' field-harvested buds entering dormancy (RfRfD; Fig. 4). The apple dehydrin gene *MdDHN2* (Liang *et al.*, 2012), on the other hand, was induced in CfCcB. Several uncharacterized putative transcription factors in apple were also DE. A predicted gene similar to *Arabidopsis AtARR9*, a response regulator involved in cytokinin signalling (Ishida *et al.*, 2008), was downregulated in both CfCcB and RfRcB comparisons. Similar results were observed with a gene highly similar to *Arabidopsis PACLOBUTRAZOL RESISTANCE 1* (*AtPRE1*), which was also induced in RfCfB as seen by microarray analysis (Fig. 4). The putative

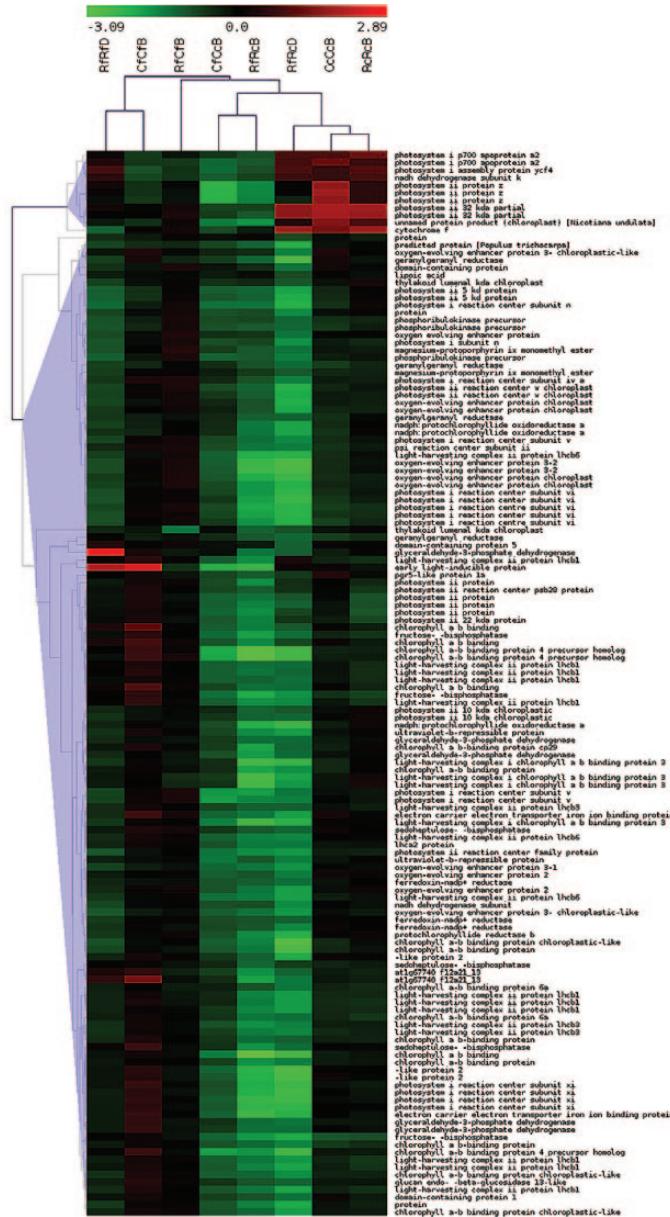


Fig. 2. Pearson correlation hierarchical clustering of log₂-transformed fold change in expression of photosynthesis-related genes. The genes shown were DE in at least one of the following manners: downregulated in RfRcD, downregulated in RfRcB, or upregulated in CcCcB. All genes were labelled with the term ‘photosynthesis’ by both Blast2GO and KOBAS, a term detected as enriched in the comparisons mentioned above by these two programs. Green and red colour intensity refer to down- and upregulation, respectively, as indicated by the bar on the top of the figure. The image was generated by MeV (Saeed *et al.*, 2003).

apple orthologue of *AtPRE1* is located at 3 Mbp from the start of chromosome 9, which is inside the LG9-QTL.

The RfRcB comparison yielded DE genes putatively encoding the main floral development regulators. In this comparison, a potential *REVEILLE1* (*RVE1*) orthologue was highly induced, as well as an *APETALA2* (*AP2*)-like transcript, while *WITH NO LYSINE KINASE* (*WNK*) was downregulated (Fig. 4). In addition to putative floral regulators, a phosphoenol-pyruvate carboxylase gene was induced in RfRcB but was downregulated after one more week of chilling (RcRcB). A chlorophyll AB binding protein gene was

repressed in RfRcB as well as in RfRcD. In this last comparison, a gene putatively encoding a *LOW OSMOTICALLY RESPONSIVE 2* (*LOS2*) orthologue was downregulated. Finally, an LHY-like transcript was upregulated in CfCcB according to microarray analysis, although real-time PCR results were not conclusive.

A gene similar to the one coding for the abscisic acid receptor PYL2 was more expressed as buds entered into dormancy (RfRcD and RfRfD; Fig. 4). In these comparisons, a gene model (MDP0000207984) annotated as FLC-like by similarity with a pear FLC-like protein, was also induced. Another gene model that was similarly annotated (MDP0000126259) was induced in ‘Castel Gala’ chilled buds (CfCcB; Fig. 4). Real-time analysis confirmed the upregulation of MDP0000126259 transcripts in CfCcB samples. Surprisingly, while microarray analysis detected no differential expression for this predicted gene in other comparisons, RT-qPCR showed that MDP0000126259 expression was significantly affected in at least five comparisons. This may be due to the low specificity for the oligonucleotide designed for this predicted transcript (data not shown), which could be hybridizing to other similar genes non-specifically. On the other hand, MDP0000207984 was confirmed as being strongly upregulated in RfRcD and RfRfD (Fig. 4).

BLAST searches in the *Arabidopsis* genome revealed no significant similarity between these genes and *FLC*, and returned other MADS-box domain-containing proteins as more similar. MDP0000126259 resembled *Arabidopsis APETALA1*, while MDP0000207984 returned MADS AFFECTING FLOWERING2 (MAF2) as the best hit, although both results yielded a relatively high expect value (0.0005 and 0.0004, respectively). *MAF2* is an *FLC* parologue with a role in avoiding premature flowering after short cold periods (Ratcliffe *et al.*, 2003). In addition to having no bona fide homologues in the *Arabidopsis* genome, BlastX searches in the NCBI non-redundant database using either gene as a query returned few significant matches (data not shown). Hence, MDP0000126259 will be referred to as *FLC-like* and MDP0000207984 as *MAF2-like*.

A contig assembled from apple EST databases and very similar to *FLC-like* can be found at the Rosaceae genome database (<http://www.rosaceae.org>, Malus_v4_Contig6253). An alignment of *FLC-like*, *MAF2-like*, and the contig is shown in Fig. 5. Both transcripts were predicted from genomic sequences anchored to apple chromosome 9 and were distanced by approximately 23 million base pairs. *FLC-like* was located approximately 700 000 bp from the start of chromosome 9, therefore lying inside the LG9-QTL.

Discussion

The complex nature of dormancy cycling is challenging, with its well-defined physiological states despite few or no morphological changes, potentially resulting from independent and synchronous molecular mechanisms (Cooke *et al.*, 2012). Because cold exposure is the main requirement for dormancy release, this work tried to determine the biological effects of cold, in the form of gene expression regulation, with respect to dormancy progression.

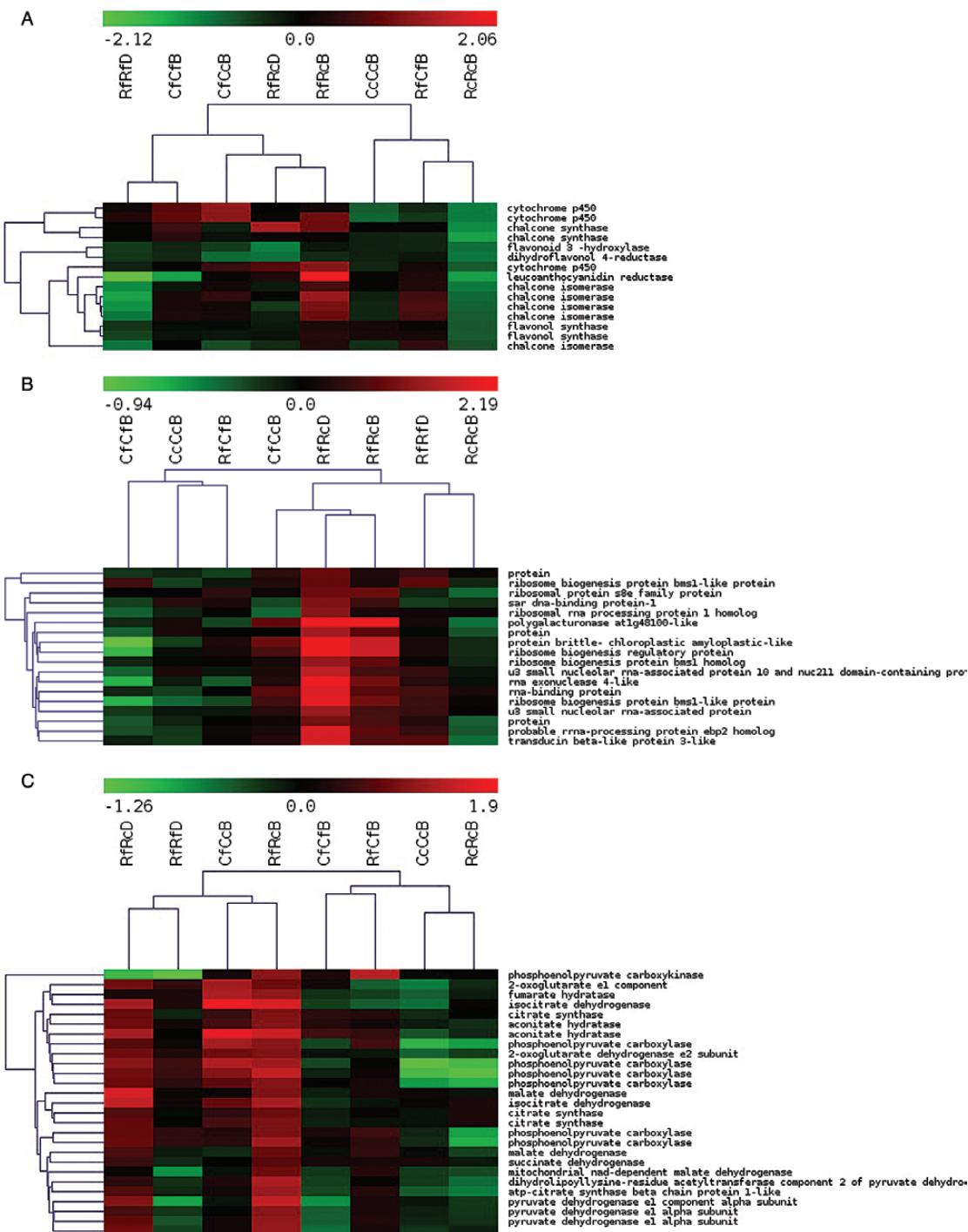


Fig. 3. Pearson correlation hierarchical clustering of \log_2 -transformed fold change in expression of DE genes classified with the term ‘flavonoid biosynthesis’ (A), ‘ribosome biogenesis’ (B), and ‘tricarboxylic acid cycle’ (C) by both Blast2GO and KOBAS. Genes in (A) were all downregulated in CcCcB, genes in (B) were all upregulated in RfRcD, and genes in (C) were all upregulated in RfRcB. Green and red colour intensity refers to down- and upregulation, respectively, as indicated by the bar on the top of the figure. The image was generated by MeV (Saeed *et al.*, 2003).

A series of hybridizations was performed comparing RNA isolated from field-harvested buds and buds chilled under controlled conditions. The dormancy status from all materials was monitored by forcing, and an attempt was made to identify transcripts associated with changes in dormancy status. Transcript profiling studies of bud dormancy usually involve comparison of samples either harvested in the field, with CR being fulfilled under

field conditions (Horvath *et al.*, 2008; Liu *et al.*, 2012) or forced to release dormancy by chilling or chemical treatments (Mazzitelli *et al.*, 2007; Gai *et al.*, 2013). This work intended to use both approaches in parallel, while taking advantage of the model ‘Royal Gala’ versus ‘Castel Gala’. Overall, this strategy deals with three sources of influence over dormancy status: cold treatments, genotype effects, and harvesting dates.

Table 3. Members of conserved miRNAs that were DE in the microarrays

Comparison codes in bold denote upregulation of the miRNA member. ARF, AUXIN RESPONSIVE FACTOR; LCR, LEAF CURLING RESPONSIVENESS.

Family	Member	Up- or downregulated in comparison(s)	Target	Reference
miR159	miR159e	RfRcB	GAMYB-like	Alonso-Peral et al. (2010)
	miR159g	RfRcB		
	miR159k	RfRcB, RfRcD, CcCcB		
	miR159p	RfRcB, RfRcD		
miR160	miR160h	RfRcD	ARF10, ARF16, ARF17	Mallory et al. (2005)
miR162	miR162a	RfRcB	DICER-like	Xie et al. (2003)
miR166	miR166a	CfCfB	HD-zip	Rhoades et al. (2002)
	miR166d	RfRcD, RfRcB		
	miR166i	RfRcB		
miR168	miR168a	RfRfD	ARGONAUTE	Vaucheret et al. (2004)
	miR168b	RfRcD		
	miR168c	RfRcB		
miR390	miR390c	RfRcD, RfRfD	ARF3, ARF4	Fahlgren et al. (2006)
	miR390d	RfRcD, RfRfD		
miR394	miR394b	RfRcD, RfRcB	LCR	Song et al. (2012)
miR399	miR399g	RfRcD, CfCcB	PHO2	Pant et al. (2008)

Day length shortening and cold treatments are known to induce and release bud dormancy, respectively, under controlled conditions. In apple, however, day length seems not to be perceived as an environmental cue to elicit growth cessation (Heide and Prestrud, 2005). In this work, we were able to induce apple bud dormancy under controlled conditions by low-temperature exposure, but the effectiveness of this treatment was highly variable. ‘Royal Gala’ buds seem to be more responsive to constant cold than ‘Castel Gala’ buds, and for both cultivars, treatments yielded the expected results more often during late autumn and winter (May, June, and July) than during early autumn (March and April). A possible reason is that apple buds are responding to environmental signals other than cold at the time of growth arrest. This may be related to the counter-intuitive finding that apple trees respond to day length when expressing a peach *CBF* gene (Wisniewski et al., 2011). Despite being part, presumably, of a cold signalling pathway, the transgene was able to connect day length perception with dormancy induction, demonstrating that mechanisms other than cold perception may act on apple bud dormancy entrance.

Cold treatments had a great impact on the apple bud transcriptome, with 3605 and 4355 genes DE in RfRcD and RfRcB, respectively, and repressed transcripts in these two comparisons were enriched in photosynthesis-related terms. Cold exposure is known to downregulate photosynthesis-related genes (Hannah et al., 2005). Nearly one-third of the genes that were DE in RfRcD (towards deep dormancy) had the same behaviour in RfRcB (towards dormancy release), indicating that many cold responses are common among buds in different dormancy stages.

The comparison that showed the lowest number of DE genes was RfCfB, which was the only one directly comparing the two contrasting CR cultivars. Similarly, differential expression screening of subtractive hybridization libraries constructed from peach and apricot bud samples during

dormancy release revealed relatively low validation rates (Yamane et al., 2008; Leida et al., 2010). These data indicate that dormancy release does not necessarily induce substantial changes in the overall transcriptional programme of buds.

Interestingly, some photosynthesis-related genes were induced in the middle and end of endodormancy. Induction of photosystem genes during endodormancy was also shown by Horvath et al. (2006) and Liu et al. (2012). Promoter analysis showed a high occurrence of the conserved G-box motif (CACGTG) and the G-box-like version (ACGTG) in non-induced photosynthesis-related genes at the end of dormancy, whereas this motif was found in only one of the induced genes. G-box sequences are bound by PHYTOCHROME INTERACTING FACTOR (PIF) 3, a transcriptional repressor that is targeted for proteosomal degradation upon light perception by phytochromes A and B (Park et al., 2004). At the end of dormancy, expression of photosynthesis genes that are not a target of PIF proteins may be derepressed after prolonged cold acclimation.

Another group of genes, related to flavonoid biosynthesis, was enriched among downregulated genes during dormancy release of ‘Royal Gala’ buds under controlled conditions (RcRcB). Most of the genes in this differentially regulated cluster, coding for chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavonoid 3-hydroxylase (*F3H*), and flavonol synthase (*FLS*), participate in flavonol biosynthesis. Flavonols are reported to inhibit polar auxin transport, which affects shoot development (Kuhn et al., 2011). Auxin levels and transport rate control the growth of dormant buds (Ferguson and Beveridge, 2009). In addition, a positive regulator of expression of flavonoid biosynthesis genes, *MAX1*, is required for the repression of axillary bud outgrowth in *Arabidopsis thaliana* (Lazar and Goodman, 2006). *MAX1* is reported to induce the transcription of *CHS*, *CHI*, *F3H*, and *FLS*, among others, resulting in enhanced apical dominance and inhibiting the growth of axillary buds. Therefore, repression of flavonoid production may be playing a role in development of apple buds after CR fulfilment.

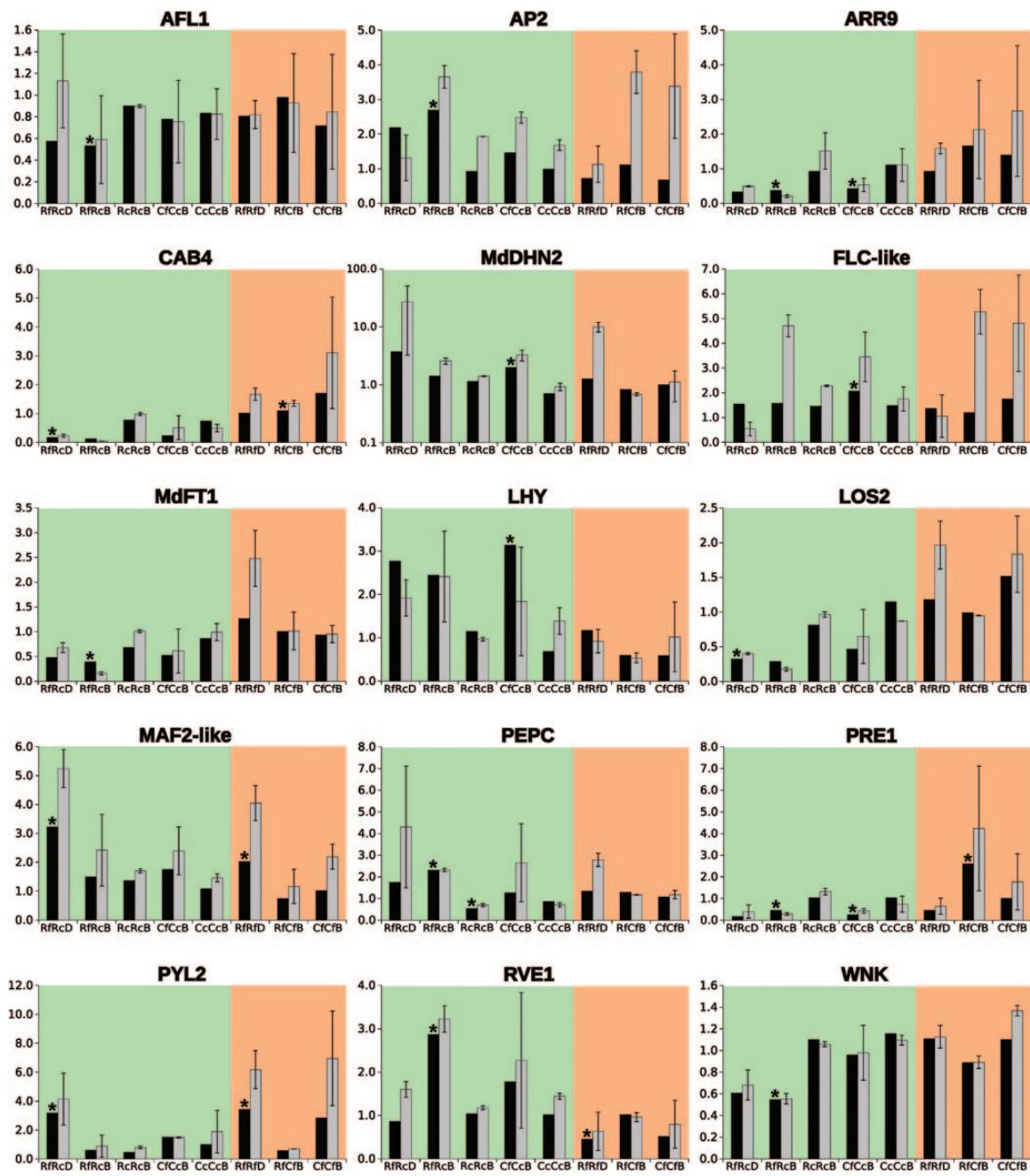


Fig. 4. RT-qPCR validation of microarray data. Values represent fold change in gene expression for selected genes by microarray (black columns) and RT-qPCR (grey columns, bars show standard error). Asterisks indicate differential expression by microarray analysis according to the adopted cut-off values (see Materials and methods). Values inside the left areas were derived from cold treatment experiments, whereas values inside the right areas were derived from samples maintained under field conditions. Comparison codes are as described in Materials and methods. (This figure is available in colour at JXB online.)

Cold treatments (RfRcB and RfRcD) also induced clusters of genes with a putative role in ribosome biogenesis (Fig. 3B). Upregulation of steady-state levels of mRNAs coding for ribosomal proteins by low temperatures was reported for soybean (Kim *et al.*, 2004), *Brassica napus* (Sáez-Vásquez *et al.*, 2000), and in dormant buds of pear (Liu *et al.*, 2012). The link between RNA metabolism and low-temperature responses has been shown by the characterization of the *A. thaliana* *low osmotically responsible (los)* mutants. Protein elongation under cold environmental conditions is diminished in *los1* plants, impairing their cold acclimation (Guo *et al.*, 2002),

while disruption of RNA export in *los4* mutant plants compromises cold signalling and tolerance to low temperatures (Gong *et al.*, 2002, 2005). Enrichment tests also detected increased transcription of genes related to the TCA in 'Royal Gala' buds exposed to cold (Fig. 3C). Transcription of genes encoding TCA enzymes were induced during chilling treatment in dormant buds of *Paeonia ostii* (Gai *et al.*, 2013) and poplar (Rohde *et al.*, 2007).

The microarray chip used in this study also featured probes designed for the detection of non-coding transcriptional information. A class of small non-coding RNAs, called

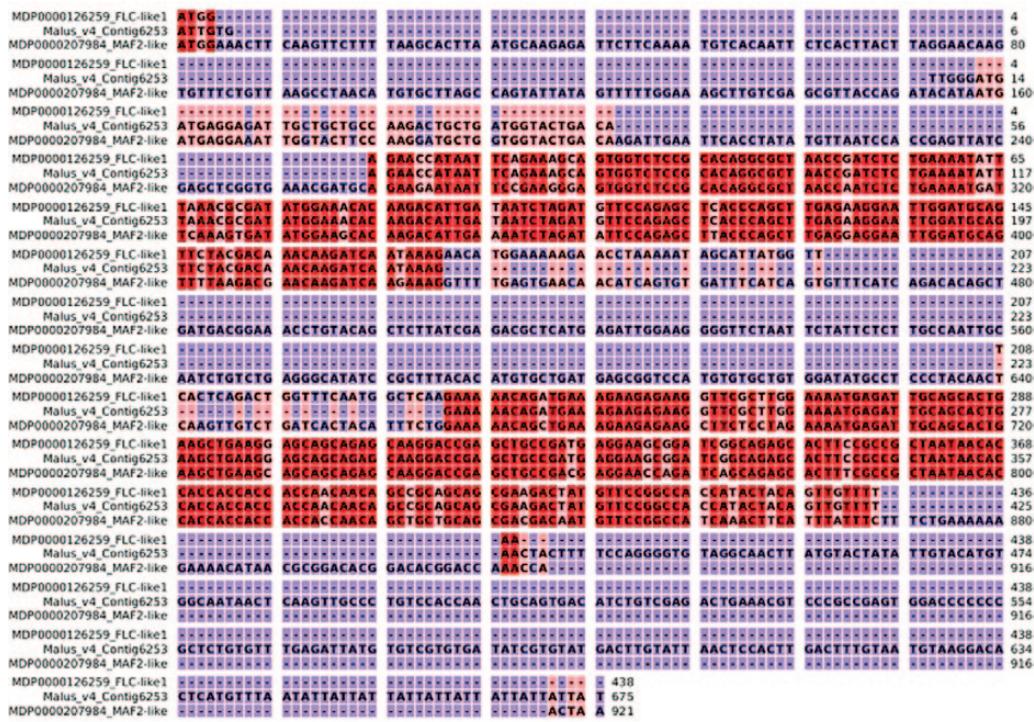


Fig. 5. Nucleotide alignment of *FLC-like*, *MAF2-like*, and contig number 6253 from the apple EST database at the Genome Database for Rosaceae. (This figure is available in colour at JXB online.)

miRNAs, is involved in development, stress responses, and other processes, and some of its members are conserved across plant families (Cuperus *et al.*, 2011). Most of the miRNA genes that were DE in the comparisons were downregulated, and at least three miRNA families represented have gibberellin and auxin signalling genes as targets, namely miR159, miR160, and miR390. Both hormones are involved in bud dormancy (Ferguson and Beveridge, 2009; Rinne *et al.*, 2011). Furthermore, miR160 and miR390 genes, which target auxin response factors, were DE only in comparisons that resulted in a decrease in bud break rate (dormancy entrance). Both miRNAs are involved in the regulation of leaf and floral organ patterning, and loss of miR390 function is reported to accelerate juvenile-to-adult phase transition (Mallory *et al.*, 2005; Fahlgren *et al.*, 2006). In addition, a miR166 gene, which was downregulated in several comparisons, is involved in the differentiation of protoxylem into metaxylem, and its absence is important for meristem maintenance (Sparks *et al.* 2013). Finally, miR394 is also involved in meristem organization, being required for the proper delimitation of the organizing centre stem cell zone (Knauer *et al.*, 2013; Sparks *et al.*, 2013). An important miRNA controlling adult phase transition, miR156, was not DE in our data but did show differential regulation in poplar (Wang *et al.*, 2011). However, many predicted homologues of its target genes *SQUAMOSA PROMOTER BINDING-LIKE PROTEIN* were DE, mainly in RfRcD and RfRcB comparisons (data not shown). This indicates that apple miR156 may be acting in buds, but this was not detected in the hybridizations.

In addition to miRNAs, regulation of gene expression by non-coding transcripts may occur via antisense RNAs (Wang

et al., 2005). In this work, approximately half of all DE genes also showed differential expression of antisense transcripts. Of these antisense RNAs, approximately half were DE in the same manner as their sense counterparts. Antisense transcription in apple was recently found in all major organs, especially in fruits and seeds (Celton *et al.*, 2014). The biological significance of these results was further shown by high-throughput sequencing of small RNAs. The number of small RNAs derived from each predicted transcript was correlated with its level of antisense expression (Celton *et al.*, 2014). The data indicated that antisense transcription is a major resource for regulating gene expression in apple. We hypothesize that antisense RNAs are actively regulating the expression of the DE genes found in this study that were positive for differential antisense transcription, and that this is particularly true for the genes exhibiting different profiles between sense and antisense expression.

Microarray results were reassessed by real-time PCR for 15 DE genes, chosen by potential relevance to dormancy and flowering time regulation and responsiveness across comparisons. The responsiveness criterion has the same rationale as the whole experiment design. It is difficult to address dormancy-related roles to genes after differential expression studies because dormancy is tightly associated with other processes such as cold acclimation and tolerance to water deficit (Cooke *et al.*, 2012). Hence, genes that are DE in several comparisons, each involving dormancy changes resulting from different conditions (chilling accumulation, genotype effects, etc.), have a higher probability of being involved with the dormancy itself, rather than with the condition responsible for changing the dormancy status.

The expression of *MdFT1* and *AFL1*, apple orthologues of *Arabidopsis FT* and *LEAFY*, respectively, was repressed in RfRcB. These two genes encode flowering pathway integrators decisive for the timing of flowering (Nilsson *et al.*, 1998; Amasino and Michaels, 2010). Transcription of *Arabidopsis FT* is known to be repressed by the action of MADS-box transcription factors such as FLC, MAF2, and SVP, which in their turn are downregulated by prolonged ambient low temperatures (Horvath, 2009; Seo *et al.*, 2009). However, the transposition of this *Arabidopsis*-based model to tree buds is not straightforward. For example, in poplar, there are two *FT* paralogues with very distinct expression patterns and roles in flowering and growth (Hsu *et al.*, 2011). Apple has two FT homologues as well, with different expression profiles (Kotoda *et al.*, 2010), and although functional studies in heterologous systems addressed similar roles to both, they may act in different contexts during growth–dormancy cycles.

Two other potential apple orthologues of flowering time controlling genes, *AP2* and *WNK*, were induced and repressed in the RfRcB comparison, respectively. In *Arabidopsis*, *AP2* is a repressor of flowering independent of *FLC* (Yant *et al.*, 2010) and is highly induced by cold (Lee *et al.*, 2005). *WNK*s are protein kinases most likely involved in the photoperiod signalling pathway, and *wnk* mutants may present early or late flowering (Wang *et al.*, 2008).

Several components of the circadian clock have been shown to participate in cold signalling and dormancy progression. In poplar, *PtLHY* was upregulated at the onset of dormancy induction by short days (Ruttkin *et al.*, 2007), and in peach a *PSEUDO RESPONSE REGULATOR 7-like* gene accumulated in dormant buds (Leida *et al.*, 2010). In poplar, disruption of *PtLHY* expression impairs freezing tolerance and delays bud burst, while *PtTOC1* silencing renders the plants more tolerant to freezing (Ibáñez *et al.*, 2010). In this work, a potential apple orthologue of *LHY* was induced during chilling-induced dormancy release. Two other clock-related genes, *RVE1* and *ARR9*, were DE as well. *Arabidopsis RVE1* is a member of the *LHY* family but is not a clock component. Rather, it functions as a clock output to control diurnal variations of auxin levels (Rawat *et al.*, 2009). Auxins have a prominent role in dormancy regulation, mainly during paradormancy (Horvath *et al.*, 2003), and also in the control of axillary bud outgrowth (Lazar and Goodman, 2006). In addition, *RVE1* was recently implicated in multiple dormancy responses in leafy spurge (Doğramacı *et al.*, 2014). Auxins also inhibit the production and signalling of cytokinins (Shimizu-Sato *et al.*, 2009). Accordingly, in the same comparison where *RVE1* was induced, a putative cytokinin-induced response regulator, *ARR9*, was downregulated. In *Arabidopsis*, *ARR9* is also clock regulated (Ishida *et al.*, 2008), contributing to the notion that bud dormancy is influenced by the circadian clock.

Gibberellin signalling is one of the major control mechanisms of dormancy cycling (Horvath *et al.*, 2003; Rinne *et al.*, 2011). The microarrays detected differential expression for a gene highly similar to *PRE1*, a transcriptional activator related to gibberellin responses (Lee *et al.*, 2006). *PRE1* expression is early gibberellin inducible and its overexpression

causes early flowering. More recently, *PRE1* was shown to be part of a regulatory module integrating brassinosteroids, gibberellin, and light signalling pathways (Bai *et al.*, 2012). This *PRE1-like* gene was DE in three comparisons and localized inside the LG9-QTL, which makes it a strong candidate for a role in the control of apple dormancy.

Despite presenting differential expression during dormancy in other studies of our group (Falavigna *et al.*, 2014; D. D. Porto *et al.*, unpublished data), *DAM* genes did not appear as DE in this work. Instead, other MADS-box genes, *FLC-like* and *MAF2-like*, emerged as promising candidates for playing roles in apple dormancy. These genes are very similar to each other (Fig. 5), and one of them, *FLC-like*, localizes inside the LG9-QTL. From all the selected candidates, the most responsive to the treatments was the *FLC-like* gene, which showed a remarkable induction towards dormancy release. A very similar result was found for leafy spurge, which showed a *MAF-like* gene highly induced in ecodormant buds (Doğramacı *et al.*, 2014). Interestingly, while *A. thaliana FLC* is highly expressed during winter, being downregulated after chilling exposure, apple *FLC-like* and leafy spurge *MAF-like* showed the opposite trend. It is important to note that ecodormant buds are, by definition, still dormant, and proper environmental conditions to grow are required. Both *FLC-like* from apple and *MAF-like* from leafy spurge may be playing roles in the repression of bud outgrowth during ecodormancy.

In this work, differential expression of apple genes across various stages of bud dormancy was assessed. DE genes of some comparisons were enriched in functional classifications, including photosynthesis, flavonoid biosynthesis, ribosome biogenesis, and the TCA. The overall data indicated that control of auxin production and transport may be an important regulatory node in dormancy release of apple buds. Several candidate genes, such as *PYL4*, *MAF2*, and *PRE1*, were DE in comparisons that had little in common but changes in dormancy status, reinforcing a potential role in dormancy. The data revealed two transcription factors, *PRE1* and *FLC-like*, as strong candidates for participants in dormancy regulation. These transcription factors may play important roles in cell division and growth under the control of internal and external stimuli.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. A detailed description of bud samples, including date of harvesting, accumulated CH and dormancy status.

Supplementary Table S2. The full list of enriched functional categories across comparisons according to both Blast2GO and KOBAS programs.

Supplementary Table S3. The expression profile of both sense and antisense transcripts for all predicted apple genes represented in the array.

Supplementary Table S4. Annotations and primer sequences for differential expression validation of selected transcripts by quantitative PCR.

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