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RESEARCH PAPER

Co-ordinated gene expression during phases of dormancy release in raspberry (*Rubus idaeus* L.) buds

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Abstract

Bud break in raspberry (*Rubus idaeus* L.) is often poor and uneven, with many of the subapical buds remaining in a dormant state. In order to determine the dormancy status of raspberry buds, an empirical measure of bud burst in a growth-permissive environment following exposure to chilling (4 °C cold storage) was developed. For cv. Glen Ample, percentage bud burst in intact canes and isolated nodes was recorded after 14 d. Isolated nodes (a measure of endodormancy) achieved 100% bud burst after ~1500 h chilling whereas buds on intact plants (combined endo- and paradormancy) required an additional 1000 h chilling. A microarray approach was used to follow changes in gene expression that occurred during dormancy transition. The probes for the microarrays were obtained from endodormant and paradormant raspberry bud cDNA libraries. The expression profiles of 5300 clones from these libraries were subjected to principal component analysis to determine the most significant expression patterns. Sequence analysis of these clones, in many cases, enabled their functional categorization and the development of hypotheses concerning the mechanisms of bud dormancy release. Thus a set of novel candidates for key dormancy-related genes from raspberry buds have been identified. Bud dormancy is fundamental to the study of plant developmental processes and, in addition, its regulation is of significant economic importance to fruit and horticultural industries.

Key words: Dormancy, forcing, gene expression, microarray, raspberry buds.

Introduction

The control of bud break through a temperature-dependent mechanism is a key ecological factor in woody perennial plant survival. In addition, important aspects of plant architecture and development are regulated by the co-ordinated regulation of bud growth (reviewed in Horvath *et al*., 2003). Thus bud dormancy is fundamental to the study of plant developmental processes. Regulation of bud break is of significant economic importance to fruit and horticultural industries. Bud break in protected raspberry crops is often poor and uneven, with many lateral buds failing to break at all (White *et al*., 1998). In order to extend cropping, current practice uses heated greenhouses, and therefore the ability to predict dormancy release becomes a major factor in early fruit production. Furthermore, such concerns are likely to become increasingly important for field production in a period of rising global temperatures (Houghton, 2005).

Bud break of many temperate fruit trees is often dependent upon exposure to a particular duration of cool temperatures (chilling) to release dormancy, followed by an appropriate temperature to permit growth in the spring (Martin, 1991). Such a mechanism prevents premature bud burst during warmer winter days that could result in subsequent frost damage. The chilling duration and the range of optimum chilling temperatures needed to release dormancy vary between species and also between cultivars within species (Fuchigami and Wisniewski, 1997). For example, raspberries have a chilling requirement of 800–1500 h below 7.2 °C, but this is variable between cultivars (Lamb, 1948; Pritts, 1996; White *et al*., 1998). Usually, before and after the endodormant period (i.e.

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when growth inhibition is regulated from within the bud itself; Lang *et al*., 1987), growth can be arrested at any time by unfavourable growth conditions. This physiological state is known as ecodormancy (Lang *et al*., 1987). Current theories of dormancy suggest that endodormancy is followed by a period of ecodormancy, where buds are held in a dormant state until temperatures rise in the spring allowing growth resumption (Lang *et al*., 1987; Faust *et al*., 1997; Arora *et al*., 2003).

Raspberry also exhibits a high degree of paradormancy caused by apical dominance, which is manifested in the typical unbranched form of the canes (Måge, 1975). Although endodormancy and paradormancy both prevent visible signs of active growth of the cane, the two types of dormancy can be distinguished experimentally by growth comparison of buds on whole canes with buds on isolated nodal cuttings (Crabbé and Barnola, 1996; White *et al*., 1999).

Phenological models of endodormancy release and subsequent bud break, based on the relationship between climate and periodic plant growth, have been formulated in the past (Hänninen, 1990; Fuchigami and Wisniewski, 1997). However, an understanding of the molecular and cellular basis of signals that control the processes of dormancy induction or dormancy release in woody perennial plants remains elusive (Dennis, 1994; Chao, 2002).

Hormonal and environmental signals exert their effect on the induction, maintenance, and release of dormancy, via activation and repression of diverse gene activities (reviewed in Olsen, 2003). Thus, the molecular study reported in this paper aims to unveil insights into the overall dormancy processes. Analysis of gene expression patterns, from the onset of dormancy in raspberry buds until the period when plants have accumulated sufficient chilling to permit bud break, will potentially provide the basis for a better understanding of the physiological processes within meristematic tissues. Important tools developed in this study include the generation and analysis of two dormancy stage-specific complementary DNA (cDNA) libraries constructed from endodormant and paradormant buds, expressed sequence tag (EST) analysis, and the development and utilization of a cDNA microarray. By combining these technologies, it was possible to monitor the activity of a large number of genes simultaneously and to identify differentially expressed genes encoding dormancy-associated proteins.

Materials and methods

Plant material

Raspberry primocanes (cv. Glen Ample) were cultivated from rootstocks in pots in unheated greenhouses. Plant material was transferred outdoors at SCRI, Dundee (56°27′ N, 3°04′ W) for hardening at the beginning of October 2002 and remained outdoors for the duration of October. At the end of October, plants were defoliated by excision of all leaves at the base of the petiole with a scalpel and transferred to cold rooms held at 4 °C in the dark with roots kept in moistened compost.

Measurement of bud dormancy status

For the measurement of paradormancy, whole canes were transferred from cold rooms to heated glasshouses maintained at 20 °C and supplied with supplementary lighting to provide 400 μE m^{-2} s^{-1} on a 16/8 h light/dark photoperiod. For the measurement of endodormancy, canes were cut into single nodes using secateurs, placed into holes cut into a polystyrene sheet, and floated on water. After 14 d, bud burst was determined (by the appearance of opened scale leaves) in the top, middle, or lower parts of whole canes or in single isolated nodes. For all isolated nodes that had received *c*. 2500 h chilling, there was close to 100% bud burst and so it was assumed that the proportion of non-viable buds was not significant. Ten canes were used for each treatment (whole canes or single nodes) and the percentage bud burst was determined at each time point.

RNA extraction

For RNA extraction, buds from the top, middle, and bottom regions of the canes were collected immediately after removal from cold rooms at 15 consecutive time points representing different degrees of chilling. Scale leaves were rapidly removed by dissection with a scalpel and the defoliated buds were immediately frozen in liquid nitrogen prior to storage at –80 °C. Approximately 1 g of frozen pooled bud material per sample was ground in liquid nitrogen. Total RNA was purified using Qiagen-tip 100 (Midi) columns (Qiagen) or the RNeasy MinElute cleanup kit (Qiagen, manufacturer's protocol, www1.qiagen.com).

cDNA library construction

Poly(A)$^+$ RNA was isolated from total RNA for the construction of the cDNA libraries using Dynabeads mRNA Purification Kit (Dynal Biotech, www.dynalbiotech.com). A cDNA library from endodormant raspberry buds ('endodormant library') was constructed using poly(A)$^+$ RNA isolated from a pool of buds collected from canes which had accumulated <1200 h in cold storage and had not yet released endodormancy (time points 1–7). The library was made using the bacteriophage lambda (λ)-based ZAP-cDNA synthesis system (Stratagene, www.stratagene.com) following the manufacturer's protocol. A second cDNA library ('paradormant library') was constructed from buds collected after the canes had accumulated >1200 h in cold storage, at which stage paradormancy dominated (time points 8–15), using the Superscript Plasmid System (Invitrogen). Randomly selected clones (384) from each library were sequenced to assess the library redundancies.

Large-scale plasmid DNA preparation

Plasmid DNA for EST sequencing and microarray construction was prepared from both libraries using Multiscreen 96-well filter plates (Millipore), as described in the manufacturer's instruction manual.

Microarray probe generation

A total of 5360 cDNA inserts were amplified by PCR using plasmid DNA template, isolated from both the cDNA libraries described above, and M13 forward and reverse primers that span the multiple cloning site of the vector. Reactions were carried out in 100 μl containing 50 ng of plasmid, M13 primers (0.5 μM each), 0.2 mM dNTPs, 2 mM $MgCl_2$, and 0.10 U μl^{-1} *Taq* DNA polymerase (Promega) in 1× PCR buffer. PCR conditions were set at 94 °C for 3 min for one cycle and then 94 °C for 30 s, 54 °C for 30 s, 72 °C

for 2 min for 38 cycles, 72 °C for 7 min for one cycle. PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen) following the manufacturer's instruction and then stored at –20 °C until required. The concentration of purified PCR products was standardized at 400–500 μg μl^{-1}.

Microarray printing

A total of 2689 'endodormant' and 2671 'paradormant' cDNA inserts amplified from library clones were prepared for spotting onto modified glass slides (amino-silane coated, Nexterion, Schott Glass Ltd.). Purified amplified cDNA (5 μl at 400–500 μg μl^{-1}) was mixed with 5 μl of 99% dimethylsulphoxide (DMSO), and transferred to 384-well print plates (Genetix). Probes were printed in triplicate (within adjacent replicate blocks, detailed in MIAME submission) onto each slide using a 48-pin robotic system (Q-array Mini, Genetix) at constant relative humidity (50%) at 23 °C. The arrays were allowed to air-dry for 10 min and the cDNA spots were immobilized following the Nexterion protocol. The quality of the printing process was checked using the SpotCheck Microarray slide QC kit (Genetix).

Microarray processing

Target RNA labelling was performed using the 3 DNA Array 900 kit (Genisphere). Two replicate hybridizations with swapped dyes for each of the 15 time points were performed in a loop-type design, with each microarray hybridized with cDNA obtained from two consecutive time points, each labelled with a different Cy3 or Cy5 dye (T1/T2, T2/T3…T14/T15, T15/T1). A second set of arrays was hybridized with the same cDNA samples, but inversely labelled (dye-swap).

For each microarray, the cDNA synthesis reactions to be compared were mixed together and hybridized to the array and washed as recommended in the labelling protocol (Genisphere).

Scanning and signal detection

Arrays were scanned with an ArrayWoRx Auto scanner (Applied Precision) at suitable exposure settings for Cy3 (595 nm) and Cy5 (685 nm) at 9.756 μm resolution, generating separate TIFF images. Exposure levels were adjusted to compensate for slight variations in labelling efficiencies. Data were acquired from images using DigitalGenome software (Molecularware), and median signal intensities and background intensities (annulus setting) were determined for the Cy3 and Cy5 channels for each spot on the microarray. Background-subtracted intensity values were imported into GeneSpring (v.6.1; Silicon Genetics), whereby data from replicate spots within each array were averaged.

Intensity-dependent normalization

Data sets for each array were normalized using the LOWESS (locally weighted scatter plot smooth) algorithm (Cleveland, 1979). Data were filtered by expression level to remove unreliable, low intensity (raw background-subtracted values less than 50) data points.

Statistical analysis

Analysis of variance (ANOVA) was performed to estimate mean response at each time point and to identify genes that gave the largest differences across all the 15 time points. ANOVA was performed on normalized values of each gene and the mean values were evaluated at each of the 15 time points, allowing for dye effects and slide effects. Data were analysed using GenStat (v.7.0; www.vsn-intl.com/index.htm).

The estimated values were subsequently investigated by principal component analysis (PCA) to detect which combinations of time points were dominant. This analysis helped to reduce the number of variables present in the normalized data set to allow further interpretation.

cDNA clone sequencing, assembly and annotation

Sequencing reactions were carried out using M13 reverse primer following the manufacturer's protocol (BigDye Cycle sequencing kit version 3.1, Applied Biosystems). Completed sequencing reactions were purified using a genCLEAN dye terminator removal plate (Genetix) prior to analysis on an ABI PRISM 3700 fluorescent DNA sequencer (Applied Biosystems).

Sequences were trimmed according to chromatogram quality criteria, using the base-calling program Phred (www.phrap.org) at a score >20, and vector-derived sequences were removed using Crossmatch software (www.phrap.org). Sequences that had fewer than 80 good quality bases after trimming were discarded.

In order to estimate the redundancy of the ESTs for both libraries, related cDNA sequences from 3′ termini were assembled as contiguous sequences using the CAP3 program (Huang and Madan, 1999) with default settings of all parameters (http://genome.cs.mtu.edu/cap/cap3.html). ESTs were defined as redundant when they exhibited more than 95% identity over aligned regions and were assembled into a single contig.

Similarities to previously identified sequences were obtained by searching public databases using the BLASTN and BLASTX tools (Altschul *et al*., 1990) for nucleotide sequence and for the deduced amino acid sequences, respectively. Local databases containing non-redundant nucleotide and protein sequences obtained from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/BLAST) were searched. e-values greater than 0.01 were considered non-significant (no match).

Sequences were locally compared with the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* database (MATDB; http://mips.gsf.de) using BLASTX. The functional categorization of sequences according to their best BLAST hit (lowest e-value score) on the MATDB was done by manual curation.

Results

Evaluation of the dormancy status of raspberry (cv. Glen Ample) buds under controlled conditions

In order to examine changes in bud gene expression during dormancy release, it was important to define the dormancy status of buds during a chilling time-course. Bud burst in raspberry primocanes was measured on whole canes cultivated in pots, showing the overlapping effect of endo- and paradormancy, and on single node cuttings maintained in hydroponic culture showing only the impact of endodormancy (Crabbé and Barnola, 1996).

As reported previously (White *et al*., 1998), bud burst in whole canes was significantly delayed in comparison with isolated single nodes, suggesting that paradormancy played a significant role in repressing bud growth in canes of Glen Ample. When exposed to less than 1100 h chilling, percentage bud burst after 14 d in a forcing environment was generally below 20%, but after 1400 h exposure nearly 100% of buds had burst after 14 d at

20 °C (Fig. 1A). Buds from the lower part of the cane showed greater variability of bud burst than those from the middle and upper parts of the cane, although they exhibited a similar overall trend. In whole canes, bud dormancy was released more gradually. After 1100 h chilling, approximately 20% of buds from the upper part of the cane had opened after 14 d in a forcing environment, a similar proportion to those on isolated nodes. However, maximal bud burst in the upper part of the cane did not occur until canes had been exposed to approximately 1900 h chilling, 500 h more than required to achieve maximum bud burst in isolated nodes (Fig. 1B). Buds from the middle and lower regions of the cane failed to achieve greater than 80% and 40% bud burst, respectively, even after receiving in excess of 2500 h chilling (Fig. 1B).

Gene profiling using targeted microarrays

To investigate changes in gene expression associated with dormancy break, a targeted microarray representing cDNA clones derived from two dormancy-related libraries in raspberry was used. RNA was isolated from defoliated bud tissue over a chilling time-course of 15 weeks. The number of chilling units was calculated for each time point (Table 1) and the dormancy status of buds for each time point determined (Fig. 1). At time points 1–7 (up to ~1100 chilling hours), buds were predominantly endodormant, whereas from time points 8 onwards (~1400 chilling hours), the buds were predominantly paradormant. Two cDNA libraries were constructed therefore; one from combined RNA from time points 1–7 (endodormant library) and one from time point 8 onwards (paradormant library). Out of 609 clones sequenced from both libraries, 24% were redundant and represented only 42 different genes whereas the remaining 76% (462/609 sequences) were singletons. The relatively low degree of redundancy suggested that randomly amplifying the inserts from several thousand clones from each library for the construction of an 'anonymous' microarray, prior to sequence analysis, would be cost-effective. Only clones corresponding to an expression pattern of interest following microarray analysis were subsequently sequenced.

A total of 2689 endodormant and 2671 paradormant amplified cDNA inserts (mean size 0.75 kb) were spotted onto modified glass slides to generate the targeted microarray. Expression of all 5360 spotted cDNAs during the entire time-course was followed on 15 arrays. Good correlation between microarray expression profiles and real-time quantitative PCR (see Supplementary Fig. 1 at *JXB* online) or RNA blotting (see Supplementary Figs 2 and 3 at *JXB* online) for a sample of cDNAs confirmed the validity of the microarray approach. The full microarray experiment has been carried out to MIAME standards and has been placed, along with the entire raw data set, into the public repository ArrayExpress (http://www.ebi.ac.uk/arrayexpress/, accession number E-MEXP-825).

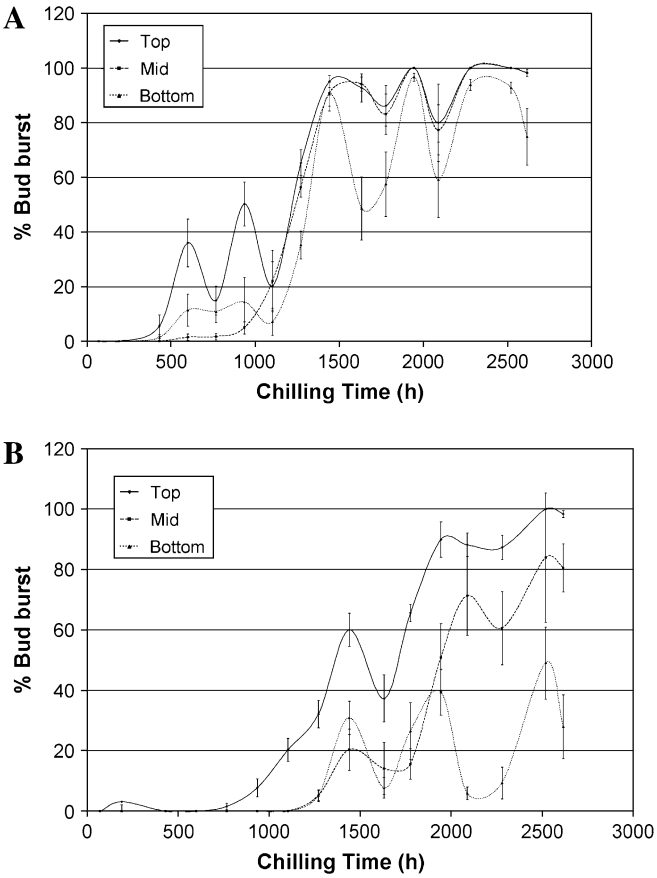

Fig. 1. Raspberry bud burst after 14 d in a permissive environment as a function of acquired chilling. At the end of an outdoor hardening period, greenhouse-grown Glen Ample raspberry canes were defoliated and transferred to vernalization rooms maintained at 4 °C in the dark. After defined intervals, canes were transferred to greenhouses maintained at 20 °C on a 16/8 h light/dark photoperiod, and bud burst in the top, middle, and lower third of each cane was scored after 14 d. (A) Bud burst in isolated nodes; (B) bud burst in whole canes. Data represent 10 biological replicates ±SE.

Table 1. *Year 1 (2002/2003): RNA extracted at 15 time points from buds collected after accumulation of a defined number of chilling hours*

Time points (TPs)	Chilling hours
TP 1	72
TP 2	240
TP 3	408
TP 4	576
TP 5	744
TP 6	912
TP 7	1080
TP 8	1248
TP 9	1416
TP10	1584
TP 11	1920
TP 12	2088
TP 13	2256
TP 14	2424
TP 15	2592

Analysis of variance

After normalization and correction of the data, the mean intensity values across pair-wise comparison of 5360 cDNAs were estimated at each of the 15 time points by performing analysis of variance (ANOVA). Once it was concluded that differences between mean values were significant (P <0.05), the nature of the variance was determined by PCA (Jolliffe, 1986). In this context, PCA of the array data from all 15 time points was performed to identify the most important expression profiles over the course of the experiment. Thus it was possible to highlight a subset of genes that were responsible for the majority of transcriptional differences and the distinct profiles underlying the differences. Three principal components were identified that together described close to 100% of the variation found in the expression patterns (Fig. 2). Principal component 1 (PC1; Fig. 2A) represented quantitative variation with no relationship to the time-course of the experiment and explained 97% of the total variability. This principal component represents constant differences in expression levels and bears no relevance to the identification of differentially expressed genes over this time-course. Principal component 2 (PC2; Fig. 2B), explaining 2% of the variability, compared early time points (prior to endodormancy release) with later time points (paradormant buds). Principal component 3 (PC3; Fig. 2C), explaining 1% of the variation, compared the mid-time points (dormancy transition) with the initial and final time points. cDNA clones clustered into PC2 and PC3 were considered to represent genes that were differentially regulated during the dormancy transition. Transcripts with the highest or lowest scores on PC2 and PC3 were selected for more detailed examination. These are the transcripts that exhibited the strongest difference in expression between the early and late stages (PC2) and between the stage of dormancy transition and the initial and final stages (PC3) of the dormancy time-course.

The selected transcripts were classified into four classes of expression pattern (ExP) as shown in Fig. 3. The 96 most differentially regulated clones (ranked on the basis of statistical significance), each from the top and bottom ends of the spectrum of variation within principal components 2 and 3, were selected for sequencing, generating 327 high quality sequences. These ESTs were subjected to

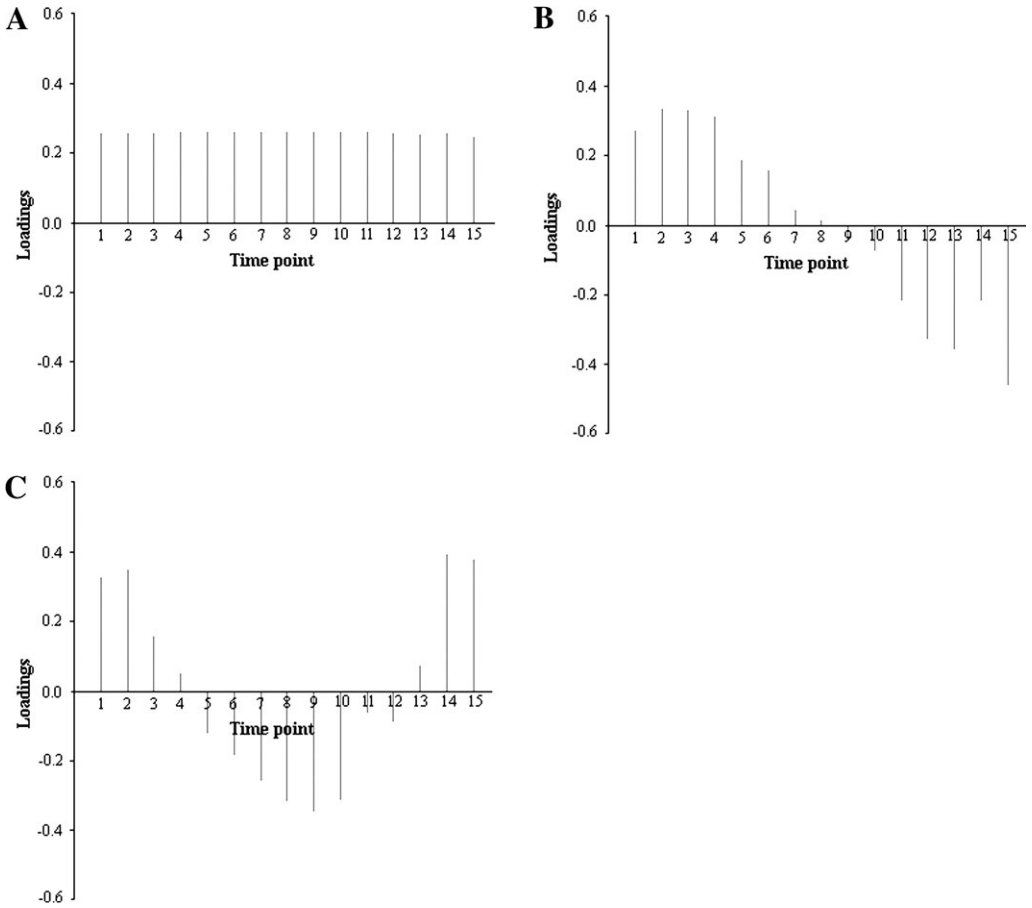

Fig. 2. Principal component loadings. (A) Principal component (PC) 1 showing scores with no meaningful relationship between different time points. (B) PC2, scores unrelated to PC1, but which show differences between early and late time points. (C) PC3 where differences were observed between the middle time points (dormancy transition) and those at either the early or late time points.

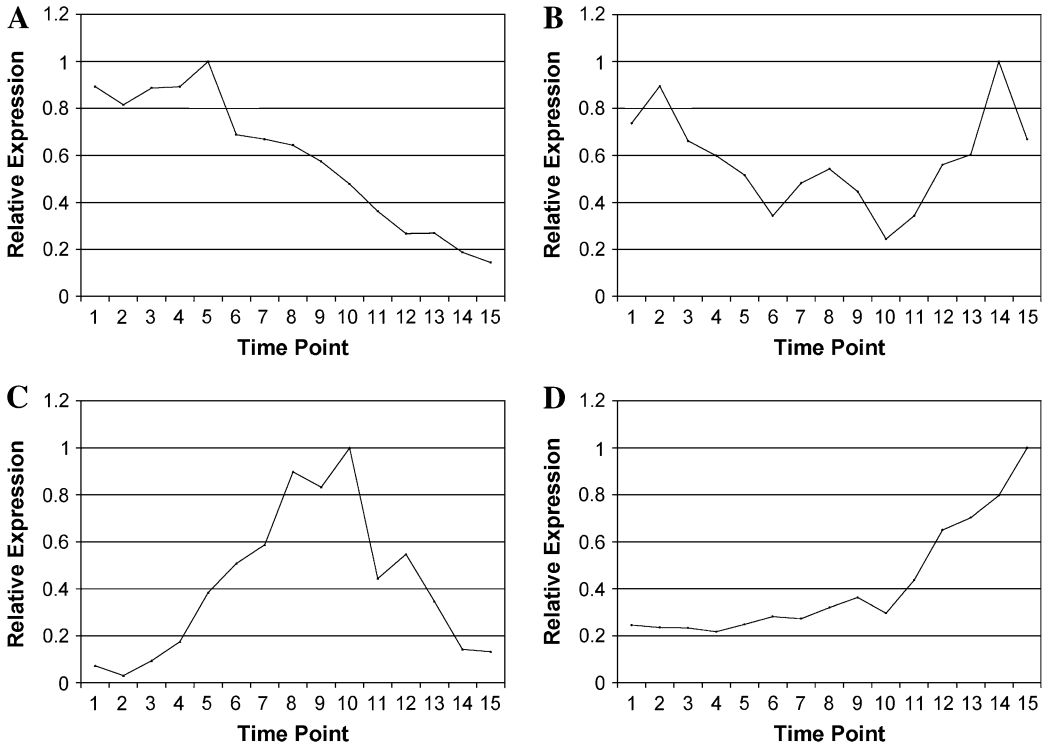

Fig. 3. Representative gene expression patterns associated with dormancy transition in raspberry buds. The data represent four examples of the different gene expression patterns (ExP) observed during dormancy transition in raspberry. (A) Rub_49 representative of ExP 1 (from principal component 2); (B) Rub_64 representative of ExP 2 (from principal component 2); (C) Rub_148 representative of ExP 3 (principal component 3), and (D) Rub_235 a representative of ExP 4 (principal component 3). Expression levels are expressed relative to the maximum observed expression level.

BLAST searches for annotation of potential function by similarity to known genes represented in publicly accessible databases (Schoof *et al*., 2002). Manual curation of MIPS MATDB functional classification resulted in the assignment of major functional classes to most of the EST clones.

Raspberry cDNAs were assigned putative functions based on their closest *Arabidopsis* homologues and, when these were unclassified or no matches were found in the *Arabidopsis* genome, matches from the NCBI database were used to provide additional information. The results of this functional classification are shown in Table 2. Over all four expression patterns, 327 good quality sequences were obtained; 7.5% were similar to unknown/hypothetical expressed proteins and *Arabidopsis* proteins to which no function could be assigned by MIPS or manual curation. Another 19% of ESTs could not be functionally characterized, but were similar to genes from species other than higher plants or gave no hit in any database (assigned as 'classification not yet clear' in Table 2). These may include novel raspberry genes or woody plant-specific genes not yet annotated in the public domain, or clones that contain insufficient coding sequence to assign precisely an annotation based on homology. Most of the putative proteins appeared to be involved in stress/detoxification responses (37.1%) followed by those participating in plant hormone responses (16.8%), cell wall metabolism (4.2%), and metabolism (3.6%). The ANOVA *P*-values, the false discovery rate (*q*-values), the average log intensity for each spot for all hybridizations, the top BLASTX result for all sequenced cloned, and the e-value for the BLAST hit as well as EMBL accession numbers and reference identification (Rub_) are available as supplementary material (see supplementary Table 1 at *JXB* online).

Discussion

A raspberry cDNA microarray was constructed and used to determine differential gene expression during dormancy release, from which specific sets of clones were selected on the basis of their expression profiles and functionally classified. Principal component analysis of the expression data enabled the identification of sets of genes with different patterns of expression correlating with changes in bud dormancy status. Several recent studies have also used this type of approach to study dormancy in other systems (Schrader *et al*., 2004; Horvath *et al*., 2005, 2006). It is interesting to compare the gene lists of differentially expressed genes from these studies and, whilst for each, many of the differentially expressed genes are not described in other species, several recurrent

themes emerge. For example, Schrader *et al.* (2004) reported much higher expression levels of LEA proteins and endochitinases in dormant cambial tissue from *Populus tremula*, compared with active tissue. In the present study these genes were also found to be expressed at higher levels in dormant buds. In recent reports (Horvath *et al.*, 2005, 2006), differential expression of genes involved in flavone synthesis during dormancy transitions were reported. Similarly, transient changes in the expression level of a gene encoding chalcone synthase were observed during dormancy release in raspberry buds.

Stress response and detoxification processes in relation to dormancy

A striking finding was the high percentage of stress-response/defence/detoxification-related genes present in all four categories of expression profiles (Table 2). This may reflect that cold acclimation and dormancy development, as well as de-acclimation and dormancy release, occur simultaneously in woody perennials (Rowland and Arora, 1997). The genes induced during the dormancy time-course suggest the activation of multiple mechanisms enabling the bud to survive low temperature. Interestingly however, each of the expression pattern categories is associated with a different set of stress-related cDNAs. ExP 1 (down-regulated during dormancy release, Fig. 3A; Table 2) was dominated by the expression of genes encoding an LEA family protein and a dehydrin. ESTs derived from ExP 2 (transiently down-regulated during dormancy release, Fig. 3B; Table 2) encoded a putative peroxidase, LEA, osmotin, plant defensins and wound-induced protein (WIP). For ExP 3 (transiently up-regulated during dormancy release, Fig. 3C), heat shock proteins (HSPs), ascorbic acid peroxidase, and osmotin were the dominant EST products. Putative metallothioneins were the most represented clones derived from ExP 4 (up-regulated during dormancy release, Fig. 3D).

Low temperature induces the production of reactive oxygen species (ROS) that can damage membrane lipids and proteins (Prasad, 1996). In fact, several studies indicate that levels of antioxidant enzymes increase in plants under stressful conditions, such as chilling or cold acclimation, in order to prevent hydrogen peroxide (H_2O_2) accumulating to toxic levels (Foyer *et al.*, 1997; Kocsy *et al.*, 2001). Furthermore, other studies indicate that bud dormancy release coincides with an up-regulation of the antioxidant system (Faust and Wang, 1993; Wang and Faust, 1994). Pacey-Miller *et al.* (2003) examined the transcript levels corresponding to antioxidant enzymes in grape buds, from the initiation of dormancy until just after the emergence of shoots, and found that these genes are mainly up-regulated after the stage of bud swelling, when oxidative damage is highly probable. Several studies report that bud break is further associated with free radical removal through activated peroxide-scavenging systems, such as catalase, ascorbate peroxidase, superoxide dismutase, and glutathione reductase (Wang *et al.*, 1991; Wang and Faust, 1994; Perez and Lira, 2005). The relative abundance of antioxidant proteins and enzymes of the ROS scavenging pathways in raspberry buds may be reflected in the expression of genes encoding ascorbate peroxidase (ExP 3, transiently up-regulated), glutathione-*S*-transferase (ExP 4 up-regulated), and glutathione reductase (ExP 4, up-regulated). It is also well known that H_2O_2 can function as a signalling molecule in plants (Foyer *et al.*, 1997; Neill *et al.*, 2002). Furthermore, Perez and Lira (2005) observed a transient increase in H_2O_2 level preceding the release of endodormancy in buds of grapevine treated with hydrogen cyanamide, a catalase inhibitor. The authors proposed that the H_2O_2 peak could act as a secondary messenger triggering the expression of genes related to endodormancy release. This may explain the high level of expression of an ascorbate peroxidase gene when endodormancy was released (ExP 3), suggesting a role in decreasing the level of H_2O_2 after it has acted as a chemical signal to induce endodormancy release.

Pla *et al.* (1998) provided evidence that HSPs and osmotin-like proteins (OLPs) accumulated in over-wintering buds of oak (*Quercus suber*). Clones with sequence similarity to HSPs were identified in the group of ESTs showing high transcript levels during the dormancy transition (ExP 3). Most HSPs function as molecular chaperones that aid adaptation to a range of internal and external stresses (Sun *et al.*, 2002). The observation of the up-regulation of numerous HSPs specifically at the dormancy transition is consistent with the large redirection of development and metabolism that occurs at this stage and the likely need to stabilize pre-existing and newly synthesized proteins in a changing intracellular environment. In addition, a gene encoding a putative OLP was highly expressed during the transition from endodormancy to paradormancy (ExP 3). Osmotin may offer some protection against the environmental stresses (such as low temperature and drought tolerance) associated with the end of endodormancy.

Bud growth, cell wall expansion, and water relations

ESTs with similarity to an aquaporin gene were found to be down-regulated at the dormancy phase transition (ExP 2). Aquaporins are proteinaceous pores involved in controlling water movement between cells (Maurel, 1997) and also possibly modulating the transport of water through plasma membranes and tonoplasts to adjust osmotic pressure. They may be a target for manipulating water flow through the plant with the potential for improving water relations and water use efficiency (Smirnoff, 1998). These proteins could possibly be related to the re-establishment of water flow in meristematic tissues after

Table 2. *List of ESTs in each functional category classified on the basis of expression pattern*

Accession numbers are available in the supplementary information at *JXB* online.

Functional category	Expression pattern 1	Expression pattern 2	Expression pattern 3	Expression pattern 4
Stress response/defence/ detoxification	Dehydrin-like protein (Rub_1–19)	Late embryogenesis abundant protein (LEA) (Rub_58)	Disease resistance protein (Rub_124)	Metallothionein (Rub_174–204)
	Late embryogenesis abundant protein (LEA) (Rub_20–26)	Metallothionein (Rub_59)	Osmotin-like protein (Rub_125–128)	Water stress-induced protein (Rub_205–210)
	Early light-induced protein (ELIP) (Rub_27–28)	Peroxidase A3 (Rub_60)	Cytosolic ascorbate peroxidase (APX) (Rub_129–132)	Plastocyanin (Rub_211–216)
	Harpin-induced protein (Rub_29)	Pyridoxamine 5′-phosphate oxidase (Rub_61)	Invertase/pectin methylesterase inhibitor (Rub_133–135)	Invertase/pectin methylesterase inhibitor (Rub_217–219)
		Wound-responsive family protein (Rub_62–63)	LMW heat shock protein (Rub_136–148)	Glutathione reductase (Rub_220)
		Defensin protein 1 (DFN1) (Rub_64–65)	Universal stress protein (USP) (Rub_149)	Glutathione *S*-transferase (Rub_221)
		Osmotin-like protein (OLP) (Rub_66–67)		Heat shock protein (Rub_222)
		Major latex-like protein (Rub_68)		Cytosolic ascorbate peroxidase (APX) (Rub_223)
		Pollen Ole e1 allergen (Rub_69)		
Metabolism	NAD-dependent sorbitol dehydrogenase (Rub_30)	Dihydroflavonol 4-reductase (Rub_70)	Leucoanthocyanidin dioxygenase protein (Rub_150)	Ribulose-phosphate 3-epimerase (Rub_224)
	Glycosyl hydrolase family 3 (Rub_31)	Chalcone synthase (Rub_71)		
	Biotin synthase (Rub_32)	Biotin synthase (Rub_72)		
	Starch phosphorylase type H (Rub_33)	Glyceraldehyde 3-phosphate dehydrogenase (Rub_73)		
		Starch phosphorylase (Rub_74)		
		S-adenosyl-L-homocysteine hydrolase (Rub_75)		
Transcription/RNA processing	MADS box transcription factor (Rub_34–35)	Phosphate-responsive protein (Rub_78)		
	DEAD box RNA helicase (Rub_36)			
	DDT domain-containing protein (Rub_37)			
	Zinc-finger protein (Rub_38)			
Energy		Phosphoenolpyruvate carboxykinase (Rub_76)		
		Ribulose *bis*phosphate carboxylase (Rub_77)		
Nucleic acid binding function			KH domain protein (Rub_151)	Small nuclear ribonucleoprotein G (Rub_225)
				Histone H2B1 (Rub_226)
				Histone H3 (Rub_227)
Protein fate	Kunitz-type protease inhibitor (Rub_39)	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (Rub_79)		Pentameric polyubiquitin (Rub_235–236)
		Cucumisin-like serine protease (Rub_80)		Ubiquitin-conjugating enzyme (Rub_237)
Protein synthesis			60S ribosomal protein L6 (RPL6A) (Rub_152)	Translation initiation factor SUI1 (Rub_228–232)
			Polyadenylate-binding protein (Rub_153)	40S ribosomal protein S6 (Rub_233)
				60S acidic ribosomal protein P2 (Rub_234)

Table 2. (*Continued*)

Functional category	Expression pattern 1	Expression pattern 2	Expression pattern 3	Expression pattern 4
Cellular transport	Sodium hydrogen antiporter (Rub_40) ABC transporter family protein (Rub_41)	Putative aquaporin TIP3 (Rub_81) Pore protein (Rub_82)	ABC transporter family protein (Rub_154–155) ATPase plasma membrane-type/proton pump (Rub_156)	
	Cyclic nucleotide and calmodulin-regulated ion channel (Rub_42)	Cyclic nucleotide and calmodulin-regulated ion channel (Rub_83)		
Plant hormone response	GAST-like gene product (Rub_44–45) ABA-inducible protein (Rub_46)	ABA-inducible protein (Rub_84–122) Auxin-responsive protein (Rub_123)	GAST-like gene product (Rub_157–166) Dormancy/auxin-associated family protein (Rub_167)	Dormancy/auxin-associated family protein (Rub_240) Gibberellin-regulated family (Rub_241)
Signal transduction	Histidine kinase (Rub_43)			SNF1-related protein kinase (Rub_238) Translationally controlled tumour family protein (TCTP) (Rub_239)
Cell wall metabolism	Basic endochitinase (Rub_47–52)		Extensin (hydroxyproline-rich glycoprotein) (Rub_168–170)	
	Polygalacturonase-inhibiting protein (PGIP) (Rub_53–57)			
Classification not yet clear	(Rub_243–267)	(Rub_268–288)	Cytochrome P450 (Rub_171) GTP-binding family protein (Rub_172) Gag/pol polyprotein – retrotransposon (Rub_173) (Rub_289–307)	GTP-binding family protein (Rub_242) (Rub_308–327)

the release of frost tolerance. However, it is not known to what degree dormancy induction may be, in part, regulated through dehydration stress via alterations in the quantity or activity of aquaporins.

Several genes involved in cell wall reorganization and synthesis were differentially expressed during the time-course of bud dormancy release. Examples include an extensin-like hydroxyproline glycoprotein, up-regulated during the time-course. By contrast, ESTs representing a polygalacturonase-inhibiting protein were down-regulated. These data may indicate that a shift in cell wall biochemistry plays an important role during dormancy release and may have some influence on the re-establishment of symplastic cell-to-cell communication through plasmodesmata that was proposed for dormancy release in birch by Rinne *et al*. (2001).

Association of dormancy release with a MADS box transcription factor

An interesting finding was the gradual down-regulation (ExP 1) during the time-course of dormancy release of a clone likely to encode an *SVP*-type MADS box transcription factor. In *Arabidopsis*, *SVP* (*SHORT VEGETATIVE PHASE*) acts as a dosage-dependent repressor of the floral transition and, consequently, its loss of function results in an early flowering phenotype (Hartmann *et al*., 2000). In accordance with its flower-repressing function, *SVP* transcriptional activity can be detected in the vegetative shoot apex, but not in the inflorescence apical meristem. *SVP* seems to exert its function independently of environmental factors such as day length and vernalizing temperatures and thus fits in an autonomous flower-repressing pathway. Interestingly, other genes that control flowering time (*CONSTANS* and *FLOWERING LOCUS T*) have also been shown to control short-day-induced growth cessation and bud set that occur in autumn in aspen trees (Bohlenius *et al*., 2006).

Genes associated with sugar metabolism

Sugar influx in the bud is generally considered to occur via H^+/sugar symports depending on the pH gradient generated by a plasma membrane H^+-ATPase (Améglio *et al*., 2000). In this study, a putative plasma membrane H^+-ATPase gene was highly expressed during the dormancy transition (ExP 3). After dormancy release in vegetative peach-tree buds, and once environmental conditions allow growth, the carbohydrate uptake capacity of the bud increases, concomitant with an increase in the expression and activity of plasma membrane H^+-ATPase (Gevaudant *et al*., 2001). These studies support

the hypothesis that, at the time of dormancy release, bud meristems require a high import of sugars from the underlying tissue, which is essential to sustain bud growth. A NAD-dependent sorbitol dehydrogenase (NAD-SDH) was represented in ExP 1 (gradual down-regulation). In plants of the Rosaceae, it has been proposed that NAD-SDH is a key enzyme in the control of sorbitol metabolism in vegetative sinks of two cultivars of Japanese pear (Ito *et al*., 2002). In fact, NAD-SDH is responsible for the oxidation of sorbitol to fructose (Loescher, 1987) and is considered important for the conversion of unloaded sorbitol to other metabolites in sink tissues.

Hormone-induced genes

Overlapping hormonal signals that are responsive to the various environmental and physiological cues that occur during the time-course of dormancy release are important in co-ordinating plant responses (Horvath *et al*., 2003). It is not surprising that several differentially regulated genes isolated in this study show similarity to sequences identified as being induced by hormones. For example, clones with similarity to a gibberellic acid-stimulated transcript from tomato (Shi and Olszewski, 1998) are highly expressed during the transition from endodormancy to paradormancy (ExP 3). These results could be a reflection of GA accumulation during the dormancy phase transition. ABA-induced proteins were identified in raspberry buds showing an ExP 2 expression profile (down-regulation during the dormancy transition), possibly indicating changing ABA levels in the bud.

Other genes of interest

A gene encoding a putative GTP-binding protein was highly expressed at the dormancy transition (ExP 3). A homologue, encoding a developmentally regulated GTP-binding (PsDRG) protein, isolated from a pea axillary bud library was observed to be preferentially expressed in growing organs such as elongating stems and growing axillary buds (Devitt *et al*., 1999). It was noted that organs which accumulated relatively high levels of PsDRG mRNA also accumulated high levels of histone H2 mRNA (Devitt *et al*., 1999). Histone mRNA accumulation is closely correlated with the S-phase and is an excellent marker for proliferating cells (Fobert *et al*., 1994). Dormancy breaking results in an up-regulation of genes that act at the G_1–S phase transition, such as histones (Horvath *et al*., 2002). In the group of ESTs categorized as nucleic acid-binding proteins, clones with sequence similarity to a histone H2 and histone H3 were present and showed a gradual increase in mRNA levels towards dormancy release (ExP 4).

Supplementary data

A table containing the ANOVA *P*-values, the false discovery rate (*q*-values), the average log intensity for each spot for all hybridizations, the top BLASTX result for all sequences cloned, and the e-value for the BLAST hit as well as EMBL accession numbers, is available at *JXB* online. Expression data from the microarrays are compared with quantitative RT-PCR data (Supplementary Fig. 1) and northern blot analyses (Supplementary Figs 2, 3).

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