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## Genes associated with the end of dormancy in grapes

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**Abstract** A grape bud EST library was constructed and 4,270 ESTs sequenced. The library clones were arrayed for the purpose of investigating the level of gene expression over time, particularly leading up to the buds' release from dormancy. The arrays were hybridized with P<sup>33</sup>-labeled probes produced from samples of buds collected at weekly intervals. These probes covered the time from 9 weeks prior to bud burst until just after the emergence of the shoots. Expression patterns from these genes have been examined. It was found that 74% of the genes in the data set were homologous to known proteins. Genes were then assigned to functional categories according to their primary BLAST match. Of these 13% were involved with photosynthesis, 13% with disease resistance and defense, 5% energy, 12% metabolism, 20% protein production and processing, 25% cell structure and plant growth and the remaining 12% were unclassified. The expression pattern of a selection of "candidate" genes retrieved from literature previously reporting an association with dormancy changes was assessed. On closer examination most of these genes relate to the oxidative processes and stress responses within the cell. The results of this study show that even in the dormant state, gene expression in the buds is high.

**Keywords** *Vitis vinifera* · Gene expression · Microarrays · Dormancy

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### Introduction

Grapevines move through a continuing cycle of dormancy and growth. Like most plants, grapes respond to the combined effect of a number of environmental variables, any one of which may restrict development by deviating from the optimum conditions for the vine. The morphology of bud and shoot development in the grape (*Vitis vinifera* L.) and other members of the Vitaceae are different from that of other woody plants. Vitaceae are characterised by a hierarchy of buds in the leaf axil (Morrison 1991). A compound "latent" bud (primary bud), is formed alongside a lateral bud within this axil. The lateral bud is vegetative and will grow on release from apical dominance (Emmerson and Powell 1978). The latent bud, however, requires a chilling period over winter before it will grow (Pratt 1974; Emmerson and Powell 1978; Pratt and Coombe 1978; Morrison 1991). This is what is considered to be dormancy in grapes.

The processes involved in plant dormancy, and the factors responsible for inducing and releasing buds from dormancy are very poorly understood and comprise a complex interrelated series of events regulated by both internal and external constituents. Environmental factors such as moisture, photoperiod, or temperature are indeed cues for all aspects of dormancy. Nevertheless, as a plant phenomenon, "regulation is genetic and expression is physiological" (Martin 1991). It is these genetic and physiological changes that need to be better understood before we can understand the dormancy process as a whole.

Many plant genes are expressed in a highly regulated manner. Gene products may be present only in certain cell types, at specific stages of development, or only following the application of distinct environmental stimuli. A single stimulus may have different effects on different genes, or a specific gene may be turned on by totally different inducers (Kuhlemeier et al. 1987). Little is known about the molecular regulation of bud dormancy in any plant. Knowing when and where a gene is expressed can give us a strong indication of its biological role. Conversely, the

pattern of genes expressed in a cell can provide detailed information about its state. Virtually all the differences that occur in the cell's state are correlated with changes in the mRNA. We can then measure the levels of mRNA associated with a particular gene by determining its cDNA sequence.

Analysis of gene expression patterns will ultimately be accompanied by a better understanding of the physiological processes within a cell. An important tool in this process has been the development of DNA microarrays. Array-based experiments aim to simultaneously catalogue the expression behaviour of thousands of genes in a single experiment (Ermolaeva et al. 1998). High-density microarrays consist of thousands of individual DNA sequences in a highly ordered matrix that can be used to measure the variation of DNA and RNA in a sample.

This study has utilised microarray technology to investigate the levels of gene expression in the buds of *Vitis vinifera* L. cv. Purple Cornichon as they approach the end of dormancy, and through the process of bud burst. It is hoped that this study will bring us closer to understanding the genetic and physiological changes behind the dormancy process.

## Materials and methods

### cDNA library preparation

Plant material was collected in Stanthorpe, Queensland, from *V. vinifera* cv. Purple Cornichon, at weekly intervals from 9 weeks before bud burst until just after the emergence of shoots. Bud swell was evident at time point 6 and bud burst occurred at time point 9 (Fig. 1). The buds were removed from the canes and pooled into a sample for that time point. These time point samples were used to make the hybridisation probes. Buds were immediately placed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction. A pooled sample from all of the time points was used to make the cDNA library for the arrays.

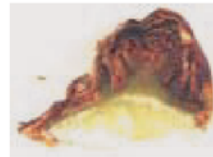
RNA was isolated from the bud material using a standard CTAB extraction and lithium chloride precipitation (Chang et al. 1993) and sent to DuPont, Newark, Delaware, United States for cDNA library construction and sequencing. Double-stranded cDNA was prepared with  $5\ \mu\text{g}$  mRNA using a Stratagene cDNA synthesis kit (no. 200401) according to the manufacturer's instructions. This resulted in cDNAs with 5' *EcoRI* and *XhoI* adapters that were ligated into *EcoRI*-*XhoI* digested pBluescript SK+. Sequencing was performed from the 5' end by DuPont (United States). Currently these sequences are held by DuPont for IP purposes.

**Fig. 1a–j** Grape buds of *Vitis vinifera* cv. Purple Cornichon showing cross sectional features of the bud. Dates of collection are given and the time point (*tp*) corresponding to later graphs is given in brackets

a. 28<sup>th</sup> July (tp1)



b. 3<sup>rd</sup> August (tp2)



c. 10<sup>th</sup> August (tp3)



d. 17<sup>th</sup> August (tp4)



e. 25<sup>th</sup> August (tp5)



f. 31<sup>st</sup> August (tp6)  
Bud Swell



g. 9<sup>th</sup> September (tp7)



h. 14<sup>th</sup> September (tp8)



i. 21<sup>st</sup> September (tp9)  
Start of bud burst



j. 28<sup>th</sup> September (tp10)  
Bud has burst



## Microarray construction

LB media was inoculated with 1 ml stock culture in LB media and incubated overnight at 37°C. Of this inoculated LB media 2.5 µl was used as the template for PCR. PCR was carried out in a total volume of 50 µl containing 4 mM Tris-HCl, pH 8.3, 20 mM KCl, 3 mM MgCl<sub>2</sub>, 10 pmol each primer M13 FWD: 5' CCC AGT CAC GAC GTT CTA AAA CG 3'; M13 REV: 5' AGC GAT AAC AAT TTC ACA CAG G 3', 0.2 mM dNTPs, 1 unit AmpliTaq Gold (Roche Applied Sciences, United States). After initial denaturation at 94°C for 10 min, 30 cycles of amplification were carried out starting at 94°C for 1 min followed by 1 min at 58°C, and 2 min at 72°C with final extension at 72°C for 5 min. Purification of the amplified product was unnecessary for use on nylon membranes.

Microarrays were prepared by printing the amplified product onto nylon nucleic acid transfer membranes (Hybond-N+, Amersham), using a robotic system (Flexys, PBA Technology) in duplicate in a 5×5 spotting pattern. The resulting array consists of 12×8 sub arrays each consisting of a square of 25 dots. This system uses a 96-pin head. A set consisting of five membranes was required to contain all genes from the cDNA library. Following printing, the membranes were allowed to dry and the DNA was bound to the membrane by denaturing in a solution of 0.5 M NaOH and 1.5 M NaCl for 1 min, neutralizing in a solution of 1.5 M NaCl and 0.5 M Tris pH 7.5 for 5 min then rinsing in 2× SSPE. The membrane was then dried briefly, but while still moist, UV-crosslinked at 120 mJ using a Stratelinker (Stratagene, Integrated Sciences, Australia). The membranes were then air-dried and stored at room temperature between Watman papers. Quality was controlled using housekeeping genes and a lambda control spot in each block.

## Probe preparation

Using a Qiagen RNeasy extraction kit (Qiagen, Australia), 400 mg bud material from *V. vinifera* cv. Purple Cornichon (Stanthorpe, QLD) was extracted following the manufacturer's instructions.

The radioactive probes were prepared from total RNA templates by incorporating P<sup>33</sup>-labeled deoxyribonucleotides during first strand cDNA synthesis in a reverse transcription reaction. RNA samples were quantified so that the equivalent of 1 µg/µl was used for each hybridisation reaction. To the lyophilised sample of RNA, 8 µl RNase-free MilliQ water was added, mixed and incubate at 70°C for 10 min.

Added to this was 4 µl 5× first strand buffer, 2 µl DTT (0.1 M), 1 µl dNTPs (minus dCTP; 10 mM) and 4 µl P<sup>33</sup>-labeled dCTP (3,000 Ci/mmol). This was mixed thoroughly and incubated at 42°C for 10 min. Next, 1 µl Superscript RT (200 U/µl; Life Technologies, Australia) was added, mixed thoroughly and incubated at 42°C for a further 50 min. Unincorporated radioactive nucleotides were removed using a Qiaquick purification kit (Qiagen, Australia). Purified products were eluted using RNase-free milliQ water. Two elutions of 25 µl were done (50 µl total).

## Hybridisation

Membranes were prehybridised at 65°C for 2 h in hybridisation buffer (5× SSC, 5× Denhardt's, and 0.5% w/v SDS).

The RNA probe solution was added to a formamide mix (93.75 µl formamide, 1.25 µl 10% SDS, 30 µl Salmon sperm DNA) and boiled for 10 min. This was then transferred to ice for 5 min. After this time the probe was added to the hybridisation solution in hybridisation bottles containing the membranes. These were left to hybridise for at least 16 h or overnight at 65°C.

Following hybridisation the membranes were rinsed with 2× SSPE:0.1% SDS at 65°C, then washed with 2× SSPE:0.1% SDS at 60°C for 20 min, then 0.5× SSPE:0.1% SDS at 60°C for 20 min twice. These were allowed to dry on Watman paper to ensure removal of excess moisture. The membranes were placed in a P<sup>33</sup> screen and exposed for 3 days. They were then scanned with the

STORM phosphoimager and the intensity of the spots measured with ArrayVision (Imaging Research, Canada). Background was subtracted using a STORM algorithm.

## Normalisation

The Total Intensity method (Hedge et al. 2000) was used for the normalisation of the set. This method takes into consideration the total intensity of all dots on one membrane compared to the total intensity of all dots on another, making this "overall intensity" value equal for all membranes. The accuracy of this method was confirmed using internal lambda controls and housekeeping genes in place throughout the membrane. The computer package Genecluster (Tamayo et al. 1999) was used to visualise gene expression patterns. This program utilises self-organising maps, a type of mathematical cluster analysis, to organise the genes into clusters with similar expression patterns.

To assure genes assessed were significant; genes that were not present in pairs were removed. That is, both points need to be significant; any points with an expression pattern that was not more than the original background plus two standard deviations of that background were removed. This was considered the significance level. If this removed one of a remaining pair then the other of that pair was also removed; on checking the graphs of the pair, these graphs should be the same. If they were significantly different they were both removed; the final set for analysis contained only those genes in a pair whose graphs were in agreement and whose intensity values were considered significant.

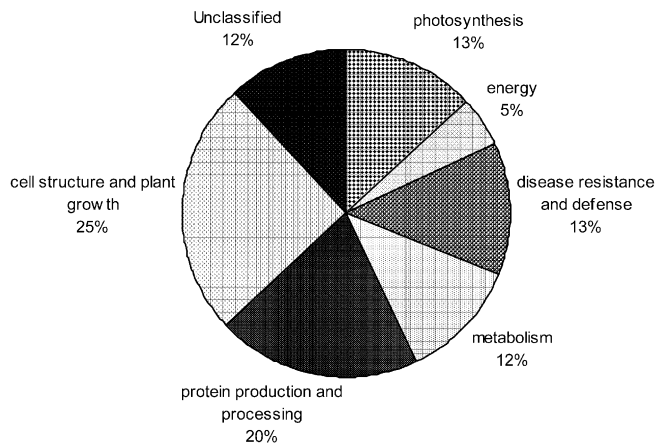
## Results and discussion

### Total expression

This study has analysed a collection of 4,270 ESTs from dormant buds of *V. vinifera* cv. Purple Cornichon. The average length of the ESTs was 424 bp. Of these cDNA's, 74% were full length or near full length, comparable to a grape cDNA library produced by Ablett et al. (2000). The samples were collected weekly over a 10-week period up to and including bud burst. Almost 26% of the ESTs matched to "unknown protein", "hypothetical protein" or "putative proteins" with no indication of the gene product. There were 382 or 9% of genes that had "no blast matches"; this class may contain novel genes or genes not yet in the public domain.

Primary blast results of those genes identified by ESTs were examined and divided into subcategories according to their cellular role. About 12% of the total set of genes assigned a blast match could not be assigned to a subcategory with certainty. An overview of the major cellular roles of the identified transcripts is given in Fig. 2. A further division was also created within these functional groups, so that a more detailed look at gene processes could be seen. This summary is given in Table 1. These values are comparable to studies done by Ablett et al. (2000).

Gene activity is present at all times throughout the time course. Because the whole metabolism of the bud is being assayed it is hard to see clear divisions between different stages of the developmental process. It is essential to examine individual genes for a more accurate determination of what is happening in the bud at any one



**Fig. 2** An overview of the major cellular roles of genes expressed during bud burst in *Vitis vinifera*. This pie graph does not include the unknown proteins, hypothetical proteins and the putative proteins that had no indication of the gene products or those with no blast match

**Table 1** A summary of the total gene pool divided into functional subcategories. Included is the percentage of the total gene pool (minus unknowns and unidentified groups) of each of the functional subcategories

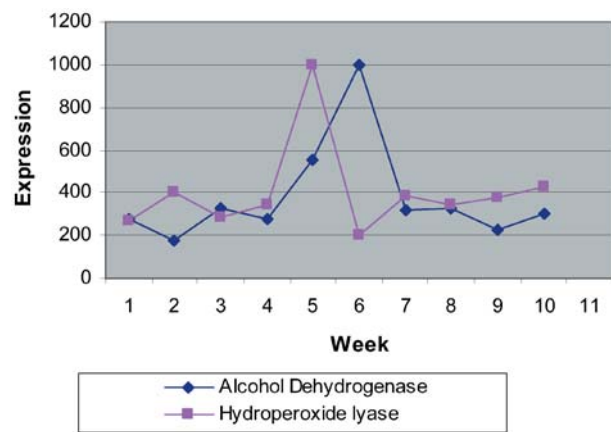
Function	%	Function	%
Defence	15	Sugar metabolism	3
Photosynthesis	14	Proteolysis	3
Cell structure	10	Krebs cycle	2
Regulation	9	Respiration	2
RNA synthesis	9	Development	2
Ribosomal protein	7	Nitrogen/sulphur metabolism	2
Amino acid metabolism	5	Other metabolism	2
Transcription factors	4	Glycolysis	1
Transport	3	Pentose/phosphate pathway	0.8
Elongation factors	3	Cell division	0.8
Lipid metabolism	3	Translation	0.4

time. Gene expression activity as a whole is very dynamic in the dormant state.

### Candidate genes

Genes that have been implicated in previous studies to contribute to the dormancy process in some way have been examined more closely to examine their pattern of expression within this data set. Expression graphs are shown in Figs. 3, 4, 5, 6, 7 and 8. Suggestion has been made in reference to the mode of action of these genes to the dormancy process. We are by no means ascribing an undue level of certainty to their function.

It has been suggested that dormancy of buds is at least partially governed by inhibition of respiration (Shulman et al. 1983). This inhibition can be removed by low temperature, cyanamide applications, and some other treatments. The mode of action of each treating agent may differ and thus also its effectiveness in breaking dormancy.



**Fig. 3** Expression of alcohol dehydrogenase and hydroperoxide lyase over time. The alcohol dehydrogenase graph shows a large peak at point 6 which corresponds to the onset of bud swell. There is generally not much activity other than this large peak. The hydroperoxide lyase graph shows a nearly fivefold increase at point 5 which is back to original levels by point 6 with very little activity throughout the rest of the time points

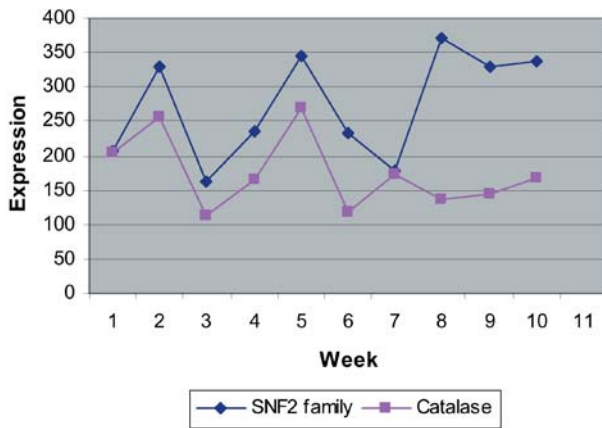
The treatment of dormant buds with chemicals such as dinitro-ortho-cresol (DNOC) and hydrogen cyanamide (HCN) results in an increase of respiration rate, as does the addition of hot water and wounding. Chilling does not increase the rate of respiration, however both chilling and the application of chemicals such as hydrogen cyanamide decrease catalase activity in the bud (Shulman et al. 1986). Hydrogen cyanamide had the greatest effect when applied just after pruning which itself induces growth (Shulman et al. 1983). Shulman et al. (1986) suggests that the release of dormancy requires oxidative processes. Dormancy breaking agents such as hydrogen cyanamide, as with chilling, indirectly increase oxidation by reducing catalase activity. Catalase decomposes hydrogen peroxide releasing stable oxygen. When catalase is reduced and peroxide levels are not, oxidative processes caused by peroxides and oxygen radicals may increase. This may in turn break dormancy.

On the addition of hydrogen cyanamide to dormant buds Or et al. (2000) noted an increase in the regulation of transcripts encoding alcohol dehydrogenase and pyruvate decarboxylase which are involved in fermentative metabolism. These results demonstrate a fivefold increase in alcohol dehydrogenase at time point 6 (Fig. 3), which is 3 weeks before the bud break event, but around the time that the bud scales are beginning to swell. This is consistent with the findings Or et al. (2000) and supports the assumption that the release of buds from dormancy involves respiratory disturbance.

There is a significant fivefold increase in the levels of hydroperoxide lyase (Fig. 3) at time point 5. This could indicate oxidative stress at this time. The plant cells are trying to break down the hydrogen peroxide in the cells, which is causing oxidative damage.

There was one transcript of the SNF2 family present in this data set (Fig. 4). SNF and SNF-like protein kinases

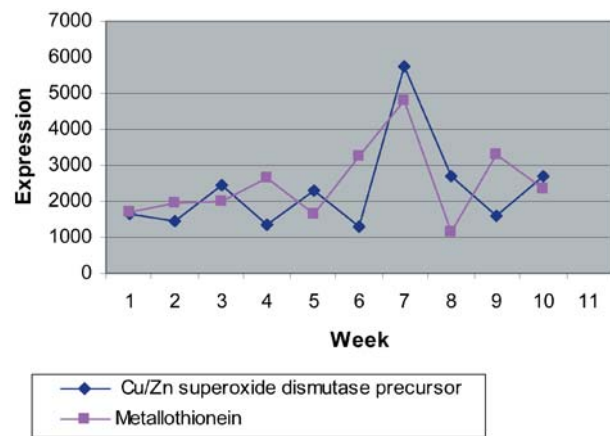




**Fig. 4** Expression of the SNF2 family and catalase over time. The expression for the SNF2 transcript shows peaks at time point 2, another just prior to bud swell at point 5 and another at peak 8 just before bud burst. The catalase expression starts with a peak rising from point 1 to point 2 then a rapid decrease to point 3. The level then increases again at point 5 (which is just prior to bud swell)

are known to be sensors of stress signals. It could be assumed that this transcript would be expressed just before any event that would present a stress to the plant. If the expression pattern of the SNF transcript is examined a rise in the levels of this transcript at time point 5, which is the time point before bud swell occurs, can be seen. This might indicate that this transcript is expressed because it has sensed the onset of a stressful environment within the cells. It would also then follow that there is a peak in the transcript just before the bud-breaking event occurs. It is unlikely that this is the trigger for these events; however the expression of SNF2 transcripts could be an indicator of stress events such as bud swell and bud burst. If this is the case then the third peak on the graph could play a significant role in discovering the trigger of the bud-breaking phenomenon. This peak occurs at time point 2, which is significantly before any physical signs of bud break can be seen.

There is a substantial reduction in the level in catalase activity from time points 2 to 3 (Fig. 4). This reduction in catalase would result in an increase in hydrogen peroxide activity. This would then be consistent with the increase in hydrogen peroxide lyase activity by time point 5. This is consistent with the suggestion that the release of dormancy requires oxidative processes. Dormancy breaking agents such as hydrogen cyanamide, as with chilling, indirectly increase oxidation by reducing catalase activity (Shulman et al. 1986). Catalase decomposes hydrogen peroxide releasing stable oxygen. When catalase is reduced and peroxide levels are not, the breakdown rate of the peroxide is reduced and the oxidative processes caused by peroxides and oxygen radicals may increase, the result being oxidative damage to the cell. The catalase levels rise at time point 5. This could be an attempt by the cell's metabolism to counteract the oxidative damage that the increase in peroxide levels is causing to the cell.

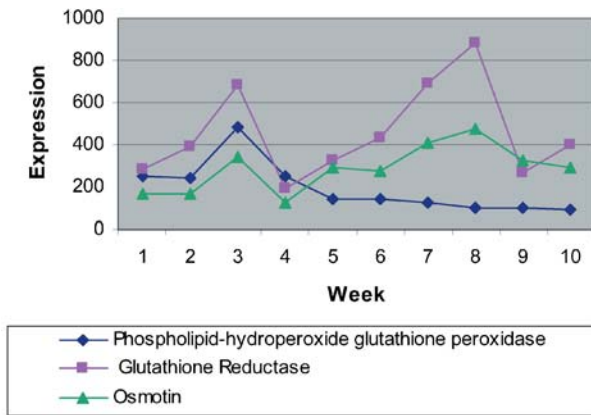


**Fig. 5** Expression of Cu/Zn superoxide dismutase and metallothionein-like protein over time. The Cu/Zn dismutase graph appears to be very similar to the metallothionein graph in that it remains relatively constant then rises rapidly at point 6 to point 7, which is immediately following the start of bud swell, then decreases to original levels towards point 9 with a slight increase at 9 at the point of bud burst. In the metallothionein-like protein graph there is minimal change in most points from time period 1 to 5 but then a significant peak in expression occurs to time period 7 which then decreases to original levels by point 8. This is occurring from the point of bud swell. There is then another small increase at point 9 which is the point at which bud burst occurs

Levels then decrease by time point 6, which is when the hydroperoxide lyase levels have also become stable.

In concurrence with the theme of oxidative damage occurring, is the presence of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR). The Cu/Zn superoxide dismutase precursors (Fig. 5) are relatively constant until time point 7 where there is a sharp peak with a sixfold variation, and they return to previous levels by point 9, which is when bud burst occurs. Foyer et al. (1997) notes an increase to cytosolic Cu/Zn SOD in response to heat shock or to chilling. This would be due to the excessive amounts of hydrogen peroxide synthesised in response to sub-optimal temperatures (Prasad 1996).

The expression of the metallothionein-like transcripts (Fig. 5) is relatively constant until a considerable peak is observed at time point 7 after bud swell, and another larger one at point 9 at the time of bud burst. The role of metallothionein is not clearly established in plants although, by analogy with animal metallothioneins, they have been suggested to play a role in metal ion detoxification or metal ion homeostasis (Tomsett and Thurman 1988). In plants, non-essential metals such as lead, cadmium, aluminium and mercury are detoxified with small peptides called phytochelatin, which are rich in the sulphur-containing amino acid cysteine and of which glutathione is a precursor (Foyer et al. 1997). They are usually only found when toxic amounts of a metal are present but are also produced when excess amounts of zinc and copper are present so they have the ability to detoxify essential metals. This demonstrates an adaptive response to environmental stress. These phytochelatin

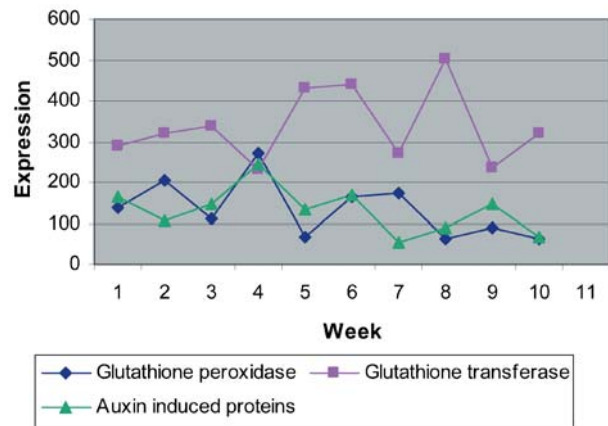


**Fig. 6** Expression of phospholipid-hydroperoxide glutathione peroxidase, glutathione reductase and osmotin like protein over time. The phospholipid-hydroperoxide glutathione peroxidase graph shows the same peak at point 3 as the glutathione reductase yet there is no second peak occurring throughout the collection period and the expression levels remain constant after decreasing to point 5 just before bud swell occurs. Glutathione reductase produced a large peak of expression at time point 3 which is still long before any signs of bud swell. There is then a steady increase up to a very large peak (fourfold increase) at point 8 which is immediately before bud burst occurs. Osmotin like protein activity is present early in the time line with a small peak at time point 3. There is a then an increase in expression up to point 8 which begins at point 6 at the onset of bud swell

act in the same way that metallothionein proteins detoxify metals in animals (Tomsett and Thurman 1988). Metallothionein-like proteins are expressed in high levels at the same point at which cytosolic Cu/Zn SOD increased (Fig. 5). This could be an example of the response mechanism to return the levels of these metal ions to a normal level after the antioxidation process has occurred.

When examining the transcripts of the antioxidant enzyme GR a threefold increase at time point 3 is observed (Fig. 6). This increase is still well before bud swell begins. There is then a steady increase that becomes slightly more rapid after bud swell until point 8. This is followed by a rapid decrease to point 9. This is in agreement with the literature in that the level has increased after the bud swell stage when oxidative damage is highly probable.

The GPXs on the other hand are at a high concentration at time period 4 (Fig. 7). The level then decreases rapidly to time point 5, just before bud swell occurs at point 6 where it increases yet again but not to levels as substantial as previous peaks. The literature indicates that antioxidant enzymes increase in plants under stressful conditions (Foyer et al. 1997). It would be expected that the levels of these enzymes would therefore be at their highest at bud swell at point 6 or at bud burst around point 9. Interestingly, however, these increases are not as high as the levels of GPX released at point 2 or more particularly at time point 4 where there is no physical appearance of change. This behaviour would indicate that although no outward signs are obvious there might be oxidative stress occurring within the dormant bud. This



**Fig. 7** Expression of glutathione peroxidase, glutathione transferase and auxin-induced genes over time. Glutathione peroxidase shows several peaks along the time course, a small one at point 2, then a much larger increase at point 4 which is still 2 weeks before bud swell. The expression then decreases below original levels but increases again during bud swell and remains at this level though the time point after bud swell. Expression then returns to original levels until bud burst when there is another very small increase. The expression of transferases also seems to be up and down throughout the length of the time course. There is a large increase the week before bud swell occurs and a peak at point 8 which is just prior to the onset of bud burst. Auxin-induced genes produce a peak at point 4 which is still 2 weeks before the start of bud swell, this decreases to lower than original levels at point 7 which is after the onset of bud swell. There is then another peak at the time of bud burst at point 9

may be an indication that the process of bud burst has begun. The observation that expression levels of glutathione peroxidase at bud swell and bud burst are not as high as previous peaks may indicate that the levels build up in the tissue after each stress encounter. There may not be a need for as much peroxidase to be released as further oxidative stress occurs.

The low molecular weight protein osmotin has been found to be formed in a high salt environment. It is suspected of helping protect the plant against salt stress (Singh et al. 1987). The expression graph of osmotin (Fig. 6) shows a peak at time point 3 and another steady increase to point 7. This increase is just before the initiation of bud burst and may offer some protection against the environmental stresses associated with the bud burst process. The peak at 3 is still well before any observable change occurs in the bud, and may indicate that there are physiological processes starting as early as time point 3 from which the cells require stress protection.

Interestingly the expression levels of phospholipid-hydroperoxide glutathione peroxidase (PHGP) shows a threefold peak at time point 3 and then the levels of the transcript decrease to almost no activity throughout the rest of the sampling period (Fig. 6). This again indicates that some sort of oxidative change is occurring within the bud at these earlier time periods. Foyer (1997) mentions that specific isoforms of glutathione transferase (GST) and glutathione peroxidase may allow rapid detoxification

of the products of lipid peroxidation generated as a result of enhanced active oxygen species (AOS) production. This phospholipid form may be more specific to the activity produced in these earlier time periods and hence no activity of this particular enzyme is indicated throughout the remainder of the time course up to bud burst.

The expression of GST (Fig. 7) shows multiple peak and trough activities. A peak is apparent at time point 8. This is just before bud burst occurs and may be in preparation for the oxidative damage occurring in the cells in order for them to expand to bud burst. There is a minor increase to point 3. This is relatively early in the time sequence yet is a pattern that is repeated in many of the other graphs we have examined. Once again this could be an indication that activity has begun as early as this in preparation for ending the dormancy process. At point 5 a considerable peak is observed. This increase in expression occurs just before bud swell where AOS production would be expected. Glutathione transferases are chimeric enzymes that catalyse the addition of glutathione to a wide range of electrophilic compounds (Marrs 1996). An example of this conjugation is the detoxification of commonly used herbicides in which the conjugates are non toxic. In addition to the GSTs protecting the plant from the outside environment by removing heavy metals, herbicides and other xenobiotics, they also play a major part in the elimination of endogenous molecules such as secondary metabolites (Foyer et al. 1997). There is obviously a lot of activity within the bud at various points over the time course where glutathione transferases are concerned and secondary metabolites may need to be removed to prevent oxidative damage at the site of the bud.

Biotic and environmental stresses lead to increases in cellular oxidants. These in turn induce an increased synthesis of non-enzymic antioxidants such as tripeptide thiol, glutathione and vitamins C (ascorbate) and E (alpha-tocopherol), as well as increases in antioxidant enzymes such as SOD, GPX, GR, ascorbate peroxidase (APX) and catalases (Foyer et al. 1997).

Thiol/disulphide exchange reactions, particularly involving the glutathione pool and the generation of the oxidant  $H_2O_2$ , are central components of signal transduction in both environmental and biotic stress (Foyer et al. 1997). Glutathione has been found to play a pivotal role in defence systems as an antioxidant and as an inducer of defence genes (Wingate et al. 1988). When the cell is depleted of GSH it is rendered susceptible to oxidative damage.

Glutathione is the major low-molecular-mass thiol compound in plants (Foyer et al. 1997) and maintains protein thiol groups in the reduced state in living cells (Tohbe et al. 1998a). Low temperatures produce an increase in ABA and oxidized glutathione (GSSG) and a gradual decrease in reduced glutathione (GSH) in grapevine shoots. Under high temperature treatment, ABA and GSSG remain constant and GSH is increased (Tohbe et al. 1998a). Tohbe et al. (1998a) also noted an increase in

GSSG content in shoots induced into dormancy by ABA treatment and that the effect of exogenous treatment with GSSH is the inhibition of bud break. In short, there is a suggestion that GSH breaks bud dormancy whereas GSSG inhibits it (Tohbe et al. 1998b). The breaking of bud dormancy could be ascribed to the conversion of GSSG to GSH, which is supposedly stimulated by cyanide produced during ethylene biosynthesis (Tohbe et al. 1998b).

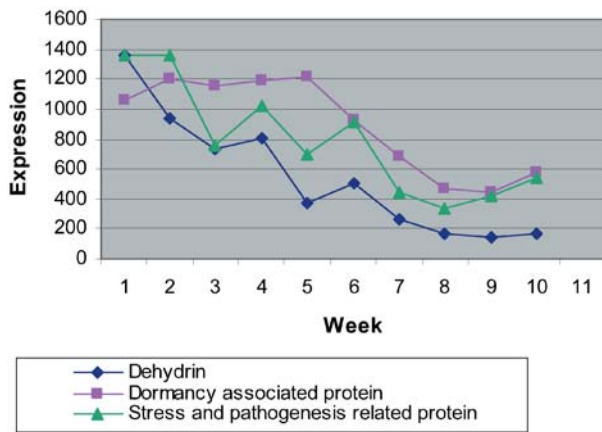
A complete understanding of the role of hormones in the control of dormancy cannot be established until we know the precise action of the hormones at a molecular level. At present we have little detail as to the nature of the dormant state at a molecular level and so the hormonal action as it relates to this stage of development remains somewhat of a mystery.

Pruning stimulates vegetative growth in a plant. Dormant pruning appears to change the metabolic equilibrium between the root system and aerial part of a plant by reducing the number of growing points which functions not only as physiological sinks but also as sources of hormones (Grochowska et al. 1984). Grochowska (Grochowska et al. 1984) suggests that severe shoot pruning increases the supply of cytokinins from the roots which triggers extensive spring growth by releasing a number of lateral buds from apical dominance. This increase in cytokinins flowing into the burst buds stimulates auxin and gibberellin synthesis, which in turn promotes further vegetative growth. Auxin induced proteins present in the data set (Fig. 7): an obvious peak at point 4 and lesser peaks at points 6 and 9. These smaller peaks correspond with the bud burst and bud swell processes. More interestingly the peak at point 4 is still at least two time points away from the start of bud swell. This is yet another indication of high gene activity before any signs of physical change are apparent in the bud.

The expression of stress- and pathogenesis-related proteins, dormancy-associated protein and dehydrin, which is released in times of water stress, is very similar (Fig. 8). This can be attributed to the fact that we may indeed be looking at a subset of the same genes. There is a general decrease in expression of all of these genes with small peaks at points 4 and 6 (at bud swell).

The change in redox state caused by respiration of the cell effects the expression of genes by affecting transcription and translation of mRNAs. Active oxygen species (AOS) have been implicated in the modulation of gene expression mediated by redox-regulated transcription in both prokaryotic and eukaryotic cells and are also considered to act as second messengers (Foyer et al. 1997). The level of  $H_2O_2$  in the plant cell at any point is determined by the relative rates of AOS production and destruction within a given compartment. AOS production is stimulated by environmental stresses such as exposure to high light, drought, heavy metals, extremes of temperature, UV radiation, air pollutants, mechanical and physical stresses, and invasion by various pathogens. AOS are therefore implicated in most, if not all, stress





**Fig. 8** Expression of stress- and pathogenesis-related proteins, dormancy-associated protein, and dehydrin over time. The overall pattern of all three graphs shows a general decrease to the occurrence of bud burst. All graphs start with a high level of expression. Dehydrin and the stress-related protein also show small peaks at points 4 and 6 (just before and during bud swell)

responses. In general, most AOS are highly reactive and lead disturbances in enzyme activity and membrane damage; as a result their generation is frequently considered to be harmful to the cell (Foyer et al. 1997). When AOS production exceeds the capacity of the tissue to scavenge them, oxidative stress is favoured. It was also found that  $H_2O_2$  increases the expression of chilling responsive genes (Prasad 1996).

Many reports have shown evidence of the existence of cross-tolerance. For example pathogenesis-related proteins and genes were found to be expressed after ozone exposure (Foyer et al. 1997). In higher plants the oxidase gene (*aox1*) has been found to have homologies with pathogenesis-related proteins (Rhoads and McIntosh 1993 cited in Foyer et al. 1997). Therefore different stressors appear to produce similar effects at a cellular level.

$H_2O_2$  and glutathione have multifunctional interactive roles in the activation of the plant stress response.  $H_2O_2$  is a strong oxidant and can cause oxidative damage in sites where it accumulates, and can diffuse a long way in the plant to cause a change in redox status in the surrounding cells and tissues. At relatively low concentrations it initiates an antioxidant response. There is no evidence that plant cells can distinguish between the different modes of  $H_2O_2$  accumulation, be it due to photorespiration or a stress response. Therefore any  $H_2O_2$  accumulation will lead to an oxidative stress response in the cellular defence systems. In this respect the plant may develop cross-tolerance to different types of stress.

Even though the bud, from an external point of view, is dormant, there is a lot of gene activity throughout the entire collection period. Many of the genes discussed have an association with the stress response in plants. This change in expression of stress-related genes could, however, be either the cause or effect of the dormancy release process. The exact moment of the start the genetic

cascade responsible for dormancy release may in fact be impossible to pinpoint.

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