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BARD

FINAL REPORT

PROJECT NO. IS-3340-02

**Exploring Mechanisms Involved in Grape Bud
Dormancy: Large-Scale Analysis of Expression
Reprogramming Following Controlled Dormancy
Induction and Dormancy Release**

E. Or, D. Galbraith, A. Fennell

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BARD Project Number: IS-3340-02R

Title: Exploring mechanisms involved in grape bud dormancy: Large-scale analysis of expression reprogramming following controlled dormancy induction and dormancy release

Investigators:

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Affiliated Institutions:

ARO, The Volcani Center

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South Dakota State University

Start Date of Project: December 1st, 2002

Date of Submission of Report: August 31, 2006

First Annual Report: December 1st, 2003

Second Annual Report: December 1st, 2004

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Keywords: oxidative stress.

Abbreviations commonly used in the report, in alphabetical order: **HC** (hydrogen cyanamide); **HS** (heat shock); **LD** (long photoperiod); **SD** (short photoperiod)

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation	2 (Or) 1 (Fennel)	1 (Fennel)	2 (Or)	6
Invited review papers (invited lecture)			1	1
Book chapters				
Books				
Master theses			2	2
Ph.D. theses			1	1
Abstracts (only international conferences)	4		2	6
Not refereed (proceedings, reports, etc.)				

*attached are 1 submitted, 2 appendixes

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Jaganatha Venkateswari (social security number 765-30-2949)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	2 (IS to AZ)	1 (SD to AZ) Fennell	3

Description of Cooperation:

The collaborative effort involved three labs with excellent interactions. The Or lab induced controlled dormancy release under three controlled systems (HC, Chilling, Heat shock), constructed the relevant libraries and supplied clones for the Vinifera EST bud collection as well as matching PCR products for array printing. The Fennell lab used a controlled photoperiod system (SD/LD) for dormancy induction, constructed the relevant libraries and supplied clones for the V. riparia EST bud collection as well as matching PCR products for array printing. Both Fennell and Or labs supplied total RNA from the various controlled systems for the hybridization steps. The Galbraith lab provided the knowledge and instrumentation for EST sequencing, microarray printing, probe production and microarray hybridizations. Dr. Or joined the Galbraith lab in July 2004 for 3 weeks for probes production and hybridizations steps and in June 2005, for final inspection of hybridization results and discussions regarding array analysis that was carried later in Drs. Or and Fennell's lab's

ABSTRACT

The timing of dormancy induction and release is very important to the economic production of table grape. Advances in manipulation of dormancy induction and dormancy release are dependent on the establishment of a comprehensive understanding of biological mechanisms involved in bud dormancy. To gain insight into these mechanisms we initiated the research that had two main objectives:

A. Analyzing the expression profiles of large subsets of genes, following controlled dormancy induction and dormancy release, and assessing the role of known metabolic pathways, known regulatory genes and novel sequences involved in these processes

B. Comparing expression profiles following the perception of various artificial as well as natural signals known to induce dormancy release, and searching for gene showing similar expression patterns, as candidates for further study of pathways having potential to play a central role in dormancy release.

We first created targeted EST collections from *V. vinifera* and *V. riparia* mature buds. Clones were randomly selected from cDNA libraries prepared following controlled dormancy release and controlled dormancy induction and from respective controls. The entire collection (7920 *vinifera* and 1194 *riparia* clones) was sequenced and subjected to bioinformatics analysis, including clustering, annotations and GO classifications. PCR products from the entire collection were used for printing of cDNA microarrays. Bud tissue in general, and the dormant bud in particular, are under-represented within the grape EST database. Accordingly, 59% of the our *vinifera* EST collection, composed of 5516 unigenes, are not included within the current *Vitis* TIGR collection and about 22% of these transcripts bear no resemblance to any known plant transcript, corroborating the current need for our targeted EST collection and the bud specific cDNA array. Analysis of the *V. riparia* sequences yielded 814 unigenes, of which 140 are unique (keilin et al., manuscript, Appendix B).

Results from computational expression profiling of the *vinifera* collection suggest that oxidative stress, calcium signaling, intracellular vesicle trafficking and anaerobic mode of carbohydrate metabolism play a role in the regulation and execution of grape-bud dormancy release. A comprehensive analysis confirmed the induction of transcription from several calcium-signaling related genes following HC treatment, and detected an inhibiting effect of calcium channel blocker and calcium chelator on HC-induced and chilling-induced bud break. It also detected the existence of HC-induced and calcium dependent protein phosphorylation activity. These data suggest, for the first time, that calcium signaling is involved in the mechanism of dormancy release (Pang et al., in preparation).

We compared the effects of heat shock (HS) to those detected in buds following HC application and found that HS lead to earlier and higher bud break. We also demonstrated similar temporary reduction in catalase expression and temporary induction of ascorbate peroxidase, glutathione reductase, thioredoxin and glutathione S transferase expression following both treatments. These findings further support the assumption that temporary oxidative stress is part of the mechanism leading to bud break. The temporary induction of sucrose syntase, pyruvate decarboxylase and alcohol dehydrogenase indicate that temporary respiratory stress is developed and suggest that mitochondrial function may be of central importance for that mechanism. These finding, suggesting triggering of identical mechanisms by HS and HC, justified the comparison of expression profiles of HC and HS treated buds, as a tool for the identification of pathways with a central role in dormancy release (Halali et al., in preparation).

RNA samples from buds treated with HS, HC and water were hybridized with the cDNA arrays in an interconnected loop design. Differentially expressed genes from the were selected using R-language package from Bioconductor project called LIMMA and clones showing a significant change following both HS and HC treatments, compared to control, were selected for further analysis. A total of 1541 clones show significant induction, of which 37% have no hit or unknown function and the rest represent 661 genes with identified function. Similarly, out of 1452 clones showing significant reduction, only 53% of the clones have identified function and they represent 573 genes. The 661 induced genes are involved in 445 different molecular functions. About 90% of those functions were classified to 20 categories based on careful survey of the literature. Among other things, it appears that carbohydrate metabolism and mitochondrial function may be of central importance in the mechanism of dormancy release and studies in this direction are ongoing. Analysis of the reduced function is ongoing (Appendix A).

A second set of hybridizations was carried out with RNA samples from buds exposed to short photoperiod, leading to induction of bud dormancy, and long photoperiod treatment, as control. Analysis indicated that 42 genes were significant difference between LD and SD and 11 of these were unique.

Achievements

Grapevine buds undergo a dormancy cycle during the winter. The timing of endodormancy induction and release is very important to the economic production of this crop. Advances in manipulation of dormancy induction and dormancy release are dependent on the establishment of a comprehensive understanding of biological mechanisms involved in bud dormancy. However, although extensive studies have been made on the physiological aspects of dormancy, there is limited information on the biochemical mechanisms and regulatory pathways involved. To gain insight into these mechanisms we initiated the current research that had two main objectives:

- A. Analyzing the expression profiles of large subsets of genes, following controlled dormancy induction and dormancy release, and assessing the role of known metabolic pathways, known regulatory genes and novel sequences involved in these processes
- B. Comparing expression profiles following the perception of various artificial as well as natural signals known to induce dormancy release, and searching for gene showing similar expression patterns, as candidates for further study of pathways having potential to play a central role in dormancy release.

To carry out these studies we developed and used controlled systems, which presents certain benefits. Short photoperiods under constant warm temperature promote rapid induction of dormancy in *V. riparia*, long before leaf fall occurs, and therefore allows us to separate induction of dormancy from the confounding effects of leaf drop and low temperature acclimation processes. Use of hydrogen cyanamide (HC), the most useful dormancy-breaking agent for grape, or heat shock (HS), provides controlled systems for analyzing dormancy release in grape buds, possessing controlled induction timing, appropriate identification for stages of development, and bud materials with relatively uniform response.

An initial step in that direction was the creation of targeted EST collections from grape buds. An EST collection from *V. vinifera* was generated from 7920 randomly selected clones that were originated from four different bud cDNA libraries (Keilin at al., manuscript): one library representing mature dormant buds sampled at different time points following controlled endodormancy release by HC and a second library representing their respective controls, a third library originated from mature buds sampled at the vineyard from mixed positions along the woody cane during the dormancy cycle and the fourth library which was constructed from young buds sampled along the shoot from bud break to veraison (Keilin at al., manuscript). A second collection from *V. riparia* was created from 1194 randomly selected clones from two mature bud cDNA libraries, one representing paradormant buds (long photoperiod, LD) and the other representing endodormant buds (short photoperiod, SD) of the same age. The entire collection was sequenced and subjected to bioinformatics analysis, including clustering, annotations and GO classifications (Keilin at al., manuscript). During the first year of our project, a large *Vitis* EST collection was created by the grape consortium and placed in the TIGR database. Towards the end of the second year this collection was used for the production of the *V. vinifera* Affymetrix microarray. Nevertheless, bud tissue in general, and the dormant bud in particular, are under-represented within these resources. Accordingly, 59% of the *V. vinifera* buds EST collection, which is composed of 5516 consensus sequences, were not formerly described for *Vitis* and are not included within the current *Vitis* TIGR collection. About 22% of these transcripts bear no resemblance to any known plant transcript, corroborating the current need for our targeted EST collection and the bud specific cDNA array (Keilin at al., manuscript). Analysis of the *V. riparia* sequences yielded 507 contigs and 207 singletons, annotated against the current NR and SwissProt databases. Expressed sequences (EST and full-length cDNA) were retrieved from NCBI for all members of *Vitis* that do not represent *V. riparia* (March 2005). About 500 *V. riparia* sequences (344 contigs, 156 singletons) had a perfect E-value (0) against non-*riparia-Vitis* sequences. There were 140 *V. riparia* sequences with no significant BLAST hit against *Vitis* (cut off E-20).

An added benefit of the *V. vinifera* bud targeted collection lies in the conferred ability to compare EST frequencies between the different cDNA libraries. Computational expression profiling of the presented EST collection was implemented to identify genes/functions whose expression is correlated with particular points in bud development or induced in response to the dormancy-release treatment. Based on this analysis, as well as following validations using northern analyses, our data suggest that oxidative stress, calcium signaling, intracellular vesicle trafficking and anaerobic mode of carbohydrate metabolism play a role in the regulation and execution of grape-bud dormancy release (Keilin at al., manuscript). These findings, which were later confirmed by the array results, led to comprehensive analysis of the potential involvement of calcium signaling in the inducing effect of HC on bud break (Pang at al., in preparation). This analysis confirmed the induction of transcription from several calcium-signaling related genes following induction of dormancy release by HC treatment and detected an inhibiting effect of calcium channel blocker and calcium chelator on HC-induced and chilling-induced bud break. It also detected the existence of HC-induced and calcium dependent protein phosphorylation activity. These data suggest, for the first time, that calcium signaling is involved in the mechanism of dormancy release.

PCR products from the entire collection from both Or's and Fennell's projects were transferred to the Galbraith lab and used for printing of cDNA microarrays (9216 spots including 152 blank spots) on amino silane-coated "Superamine" slides (Appendix A).

The development of the microarray infrastructure gives us the unique and powerful capability to compare the expression profiles following the perception of alternative dormancy breaking signals. The identification of genes having similar expression profiles following application of alternative signals may become a potent tool for the identification of pathways with a central role in dormancy release. To examine this approach and set up such system, we compared the effect of heat shock (HS) to the effect of HC and confirmed that in our experimental system HS lead to bud break which is earlier, compared with HC treatment (Halali at al., in preparation). We than analyzed the effect of HS on the expression profiles of three genes whose expression pattern was influenced by HC application. Similar to the effect of HC treatment, HS led to reduction in catalase transcript level and increase in alcohol dehydrogenase and pyruvate decarboxylase transcript levels. The changes in all transcripts levels were temporary after both HS and HC and recovery was detected after 1-2 days. While the pattern of changes was similar for both treatments, timing of change differed and changes occurred earlier in HS treated buds. These earlier temporary changes following HS were correlated with earlier bud break following this treatment. We then found that expression from ascorbate peroxidase, glutathione reductase, thioredoxin and glutathione S transferase were also induced by both treatments (Halali at al., in preparation). Altogether, these findings support the assumption that temporary oxidative stress and respiratory stress are part of the mechanism leading to bud break, and suggest for the first time that identical mechanisms are triggered by different induction signals.

Supported by this analysis, total RNA was prepared from HC, HS and control treated bud pools that were collected at six time points in three different years (Halali at al., in preparation). A second set of hybridizations was carried out with RNA samples originated from buds collected at seven time points during controlled chilling induction of dormancy release and a third set of hybridizations was carried out with RNA samples from buds exposed to short and long photoperiods treatments and sampled at four time points. PolyA RNA preps were purified from total RNA and used as templates for amplification reaction. Direct labeling of the resulted aRNA by random priming was used for the production of Cy3 and Cy5 probes. For the artificial dormancy release each time point was hybridized competitively with the next time point from the same treatment in a loop design and all time points from both treatment groups were hybridized against their corresponded time points from the control group. This design fulfilled requirements for technical replications (3 per probe) and balanced dye swap and was repeated with two biological replications from different vineyard in different years (total of 66 slides).

The redundancy that was inserted into the microarray, due to the random selection of clones for the sequencing project (Keilin et al., manuscript), allowed us to inspect and confirm the excellent technical reliability of the signal throughout the array.

Differentially expressed genes were selected using R-language package from Bioconductor project called LIMMA. This package allowed us better variance estimation by empirical Bayesian techniques, which is needed due to the small number of biological replicates and the use of loop design (See appendix A).

Based on the experiment rational, clones showing a significant change following both HS and HC treatments, compared to control, were selected for further study. Overall, 1541 clones showed significant induction and 1452 clones showed significant reduction of transcript level at one time point or more after both HS and HC treatments. Apart from the clones that had no hit or were annotated as "unknown function" (367 and 215 induced + 451 and 230 reduced, respectively) the clones represent 661 induced genes and 573 reduced genes involved in 445 and 447 different molecular functions (named "unifunction"), respectively. About 90% of the induced "unifunctions" were classified to 20 categories based on careful survey of the literature and the analysis of the reduced function is ongoing. Among other things, it appears that carbohydrate metabolism and mitochondrial function may be of central importance in the mechanism of dormancy release and studies in this direction are ongoing (see Appendix A).

For the analysis of expression profiles following controlled dormancy induction, four replications of *V. riparia* collected from age matched buds under LD (paradormancy maintenance) or SD (endodormancy induced) at 2, 3, 4, and 6 weeks of treatment were used. Arrays were hybridized in loop design with balanced dye swap. After normalization for nonlinear dye*intensity interaction effect and a global normalization for dye, array, and block effects, 8665 genes were analyzed for treatment interaction and main effects. No significant interaction between time and photoperiod was detected. Analysis of photoperiod main effect indicated that 42 genes were significantly expressed between LD and SD and ¼ of these were unique. Analysis of time main effect indicated that 839 genes were significantly expressed and cluster analysis is ongoing; however a high portion of the most significant genes are unique to *Vitis* bud or of unknown function. These unique genes are being further characterized for tissue and phenotype specificity.

For controlled chilling induction of dormancy release one loop consisted of 7 time points was designed with balanced dye swap. At present we completed hybridizations for only one out of three biological repeats that are now available. Hybridization steps, as well as analyses, are now ongoing.

Agricultural impact

Based on the results discussed above, HS appear as a cheaper and safer option for artificial dormancy release, compared with HC. Recently, we acquired funds to collaborate with agricultural engineering experts and develop applicative means for the use of HS as a dormancy release agent in the vineyard. First year results support it feasibility.

Details of Cooperation

The collaborative effort involves three labs with excellent interactions. The Or's lab induced controlled dormancy release under three controlled systems (HC, Chilling, Heat shock), constructed the relevant libraries and supplied clones for the *Vinifera* EST bud collection as well as matching PCR products for array printing. The Fennell lab used a controlled photoperiod system (SD/LD) for dormancy induction, constructed the relevant libraries and supplied clones for the *V. riparia* EST bud collection as well as matching PCR products for array printing. Both Fennell and Or labs supplied total RNA from the various controlled systems for the hybridization steps. The Galbraith lab provided the knowledge and instrumentation for EST sequencing, microarray printing, probe production and microarray hybridizations. Or joined the Galbraith lab in July 2004 for 3 weeks for probes production and hybridizations steps and in June 2005, for final inspection of hybridization results and discussions regarding array analysis that was carried later in Drs. Or and Fennell's lab's.

Appendix A: Microarray printing, hybridization and data analysis of dormancy release experiment

Microarray printing

The entire collection from both Or's and Fennell's projects were transferred to 384 well microplates. The plates were dried in a speedvac at 50°C. To each well 12ul of 3xSSC was added and transferred to orbital shaker (Belly Dancer: Sorvall Life Sci.) for 1h at RT. The grape cDNA microarrays were printed on amino silane-coated "Supramine" slides (Telechem), using Telechem Type SMP3 printing pins.

Printing conditions: Single array element printed per clone, 32 pins in the print head, 30% relative humidity and redipping after every 40 slides. Print head velocity is set to a maximum of 10mm/sec.

Array Information:

Date Printed	04-16-04
Clone Set	<i>Vitis vinifera/Vitis riparia</i> EST set
384 plates used	VVPE1- 24
Print run number	1
Grid Properties	32 grids; 18rows x 18columns (two rows 17-18 empty)
Total Spots	9216 (including 152 blank spots)
Spot Diameter	110u
Spot spacing	230u
SubArray spacing	460u
Machine used	GeneMachines™ 'OmniGrid 100'

Microarray hybridizations

Quality control of printing and hybridization protocol:

For quality control we performed hybridizations using Cy3 labeled random 9-mer for the first and last array at every print run. Slides were scanned immediately after hybridizations and stored in a light proof container in a cool dry place. Data were extracted from scanned images using Acuity 3.0. and the number of spots flagged over the total number of spots was calculated.

To test the hybridization protocol we conducted hybridizations of 5 slides with Cy5-labeled cDNA from polyA RNA from *V. vinifera* young leaf and bud samples. Based on the quality of these slides we moved forward to the hybridization step.

Total RNA samples were prepared from buds pooled at 6 time points from control (C), Heat shock (HS) and hydrogen cyanamide (HC) treated cans. PolyA RNA preps were purified and used as templates for amplification reaction. Direct labeling of the resulted aRNA by random priming was than used for the production of Cy3 and Cy5 probes. Probe pairing was carried out according to a hybridization plane that was designed under the supervision of a statistician from the U of A, Dr. David Henderson. Each time point was hybridized competitively with the next time point from the same treatment in a loop design and all time points from both treatment groups were hybridized against their corresponded time points from the control group (Fig 1). This design fulfilled requirements for technical replications (3 per probe) and balanced dye swap and was repeated with two biological replications from different vineyard in different years.

Slides were scanned, using Axon scanner and raw data, which appeared to be of overall high quality, were extracted using GenePix and subjected to statistical analysis. The size of experiment and the described design presented a complicated challenge and we invested considerable amount of time and efforts on preliminary work before we decided on final strategy of data analysis.

Figure 1: Hybridizations program (C) =control; S= heat shock; H = hydrogen cyanamide)

Differentially expressed genes were selected using R-language package from Bioconductor project called LIMMA (Linear Models of MicroArray Analysis). This package allowed us better variance estimation by empirical Bayesian techniques, which is needed due to the small number of biological replicates and the use of loop design. In short, although hybridizations were carried out with a dual channel platform, each channel was analyzed separately as described in the user LIMMA guide chapter 9. First, we weighted bad spots by 0.1 and subtract the local background measurement. This was followed by per-tip LOWESS normalization within slides and variance stabilization normalization (VSN) among slides. Technical replicates were averaged and then we estimated the intra spot correlation between the two channels of the same RNA sample and as weighting for further fitting of biological groups (eg. H3, C3 etc.). This technique is equivalent for randomize block over dye effect within spots. Finally, due to the small number of replicates, biological-comparisons variance was corrected by empirical Bayesian method to obtain moderated-t statistic and multiple comparison correction were applied. Data yielded following multiple comparison correction using FDR are presented. Figure 2 presents the number of genes showing a significant change in a given time point after either heat shock (HS) or hydrogen cyanamide (HC) treatment. From this overview it is clear that hydrogen cyanamide affected the transcriptional level of higher number of genes compared to heat shock. Moreover, in accordance with the data from our biological experiments (Halali at al., in preparation) it is apparent that heat shock effect transcription as early as 6h after treatment while the majority of changes induced by HC are evident from 12h and later. For both systems it can be seen that the effect is temporary and is reduced after 24-48 h. The agreement of these data with our previous biological knowledge proves the quality of hybridization results as well as quality and informative nature of the statistical analysis results.

Figure 2: The number of clones presenting significant increased (BHU) or decreased (BHD) transcript level compared to its control in a given time point after either heat shock (HS) or hydrogen cyanamide (HC) treatment.

The redundancy that was inserted into the microarray, due to the random selection of clones for the sequencing project (Keilin et al., manuscript), allow us to inspect the technical reliability of the signal throughout the array, and the biological reliability of the expression pattern for a single gene. An example is presented, where 10 different clones representing the same thioredoxine gene but spotted in different regions of the array show almost identical pattern of hybridization, as was also confirmed by Northern blot (Fig. 3).

Figure 3: The effect of HC and HS treatments on the expression pattern from thioredoxinH gene in the buds. Upper panel presents the averaged signals for 10 clones of thioredoxinH (TC25169) clones in the cDNA array. Lower panel presents northern analysis that was carried out with a ThioredoxinH probe (TC 25169).

Based on the experiment rational, clones showing a significant change following both HS and HC treatments, compared to control, were selected for further study. Overall, 1541 clones showed significant induction and 1452 clones showed significant reduction of transcript level at one time point or more after both HS and HC treatments. These clones lists are composed from clones that has no hit and are considered grape specific at this point, clones that are similar to genes with unknown function from other organisms and clones that show similarity to genes with known function (termed as "Informative" annotation):

Annotation	Induced clones	Induced genes	Reduced clones	Reduced genes
"Informative" annotation	959	661	771	573
Unknown function in other organism	215	198	230	169
<i>Vitis</i> specific	367		451	
TOTAL	1541		1452	

At current stage of analysis the clones annotated as unknown function from other organisms represent 198 induced and 169 reduced genes. The clones with the informative annotations represent 661 induced genes and 573 reduced genes and involved in 445 and 447 different molecular functions ("unifunction"), respectively.

About 90% of the induced "unifunctions" were classified to 20 categories based on careful survey of the literature, and their relative part (%) within the total "unifunction" list is presented in figure 4. Similar analysis is on going for the reduced functions.

Figure 4: Relative portion of each functional category from the total list of functions induced by both HS and HC

Transcription profiles of several clones that were analyzed at earlier stage (Keilin et al., Pang et al., and Halali et al. manuscripts) were in agreement with their expression profile in the array, therefore validating the array results. Among other things, it appears that carbohydrate metabolism and mitochondrial function may be of central importance on the mechanism of dormancy release and studies in this direction are ongoing. A role for sugar-sensing systems and sucrose synthase in regulation of plant-hormone biosynthesis and sensing in apical meristem development was recently reported. In this context, the concurrent and significant up regulation of both carbon metabolism and plant hormone functions will be further studied. Additionally, a collection of affected kinases (Figure 5) was selected for protein interaction studies that may expose cellular functions that are regulated during dormancy release.

Figure 5: Expression profile of selection of Kinase genes presenting significant induction following the application of heat shock (S), hydrogen cyanamide (H) and water (C).

Normalized signals for different time points for the entire collection were incorporated into GeneSpring software and were used for further analyses. Preliminary clustering analysis was carried for the clones with identified function and yielded 9 clusters for induced genes and 3 clusters for reduced genes. Figure 5 supply graphic representation of selected clusters of genes presenting significant change following both HS and HC as well as the earlier and milder effect of HS compared to HC treatment. Improvement of clustering is ongoing and clusters are subjected to a through analysis of links between different functions presenting similar expression pattern. AD library was already prepared for protein-interaction study by yeast two-hybrid technology. Due to the wealth of data, the array analysis is still ongoing and upon completion it will sumerized and reported.

Figure 6: Clusters of genes showing a significant change following both HS and HC treatments, compared to control. Selection of products from Gene Tree clustering (standard correlation) of lists of induced (959) and reduced (771) clones that show similarity to genes with known function.

Appendix B: Photoperiod induction of dormancy

V. riparia is rapidly induced into endodormancy upon exposure to short photoperiods (SD, 8h) at 22°C. A long photoperiod (LD, 15h) inhibits dormancy and maintains the buds in paradormant state. Two cDNA libraries were constructed using RNA from age matched paradormant and endodormant buds of *V. riparia* and 5' and/or 3' ESTs were generated. Analysis of 528 random paradormant cDNAs yielded 844 nonvector ESTs of a minimum of 50 high-quality bp and an average length of 590 bases. Endodormant cDNAs (666) yielded 1194 fragments with an average length of 566 bases. Sequences were published to NCBI and then processed for cluster analysis at PlantGDB (<http://www.plantgdb.org>). Clusters were generated using PaCE. The consensus sequences for each cluster were generated using CAP3. Contigs of two or more EST clusters and singletons (ESTs not significantly similar to other ESTs) were identified. Contigs and singletons were annotated against the March 2005 NR and Swissprot databases. Expressed sequences (EST and full-length cDNA) were retrieved from NCBI for all members of *Vitis* that do not represent *V. riparia*.

Potted, spur-pruned *V. riparia* vines were removed from cold storage and grown under a LD (15 h) at 25/20 + 3°C day/night temperatures (D/N) as previously described (Fennell, et al., 1996; Fennell and Mathiason, 2002). After 45 days of growth, uniform vines with 3 or 4 shoots that are 10 to 12 nodes long, were randomly assigned to the SD (8 h, dormancy inducing) or LD at 25/20 + 3°C D/N in climate controlled greenhouse with automated photoperiod system (VRE Greenhouse Systems). One hundred eighty vines were assigned to each photoperiod. All treatments were repeated at the same time in each of four years and harvested at the same time of day each year to minimize biological noise and generate four replications. At 2, 3, 4 and 6 weeks of LD or SD treatment, buds were harvested from nodes 3 to 12, immediately frozen in liquid nitrogen, and placed at -80°C for future RNA extraction (Fennell et al., 1996; Fennell and Mathiason, 2002). After photoperiod treatments and bud harvests, all pruned vines were returned to LD and monitored for bud endodormancy. The endodormant vines are identified after 28 days in LD and moved to cold storage. The nondormant vines are allowed to grow again and induced into dormancy at a later date. For the analysis of expression profiles following controlled dormancy induction, four replications of *V. riparia* collected from age matched buds under LD (paradormancy maintenance) or SD (endodormancy induced) at 2, 3, 4, and 6 weeks of treatment were used. Arrays were hybridized in loop design with balanced dye swap. After normalization for nonlinear dye*intensity interaction effect and a global normalization for dye, array, and block effects, 8665 genes were analyzed for treatment interaction and main effects. No significant interaction between time and photoperiod was detected. Analysis of photoperiod main effect indicated that 42 genes were significantly expressed between LD and SD and ¼ of these were unique. Analysis of time main effect indicated that 839 genes were significantly expressed and cluster analysis is ongoing; however a high portion of the most significant genes are unique to *Vitis* bud or of unknown function. These unique genes are being further characterized for tissue and phenotype specificity.

Exploring dormancy release with a unique grape-bud EST set

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Computational expression profiling of a grape-bud EST collection leads to novel insight into molecular and cellular events during grape-bud dormancy release.

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Footnotes

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ABSTRACT

The application of genomic approaches may serve as an initial step in broadening our understanding of the complex network of biochemical and cellular processes responsible for the regulation and execution of grape-bud dormancy release. However, bud tissue in general, and the dormant bud in particular, are under-represented in the current public *Vitis* genomic resources. Here we describe the first **public** effort to generate a large-scale grape-bud EST collection representing a wide range of bud developmental stages. A collection of 5516 consensus sequences is presented, of which 59% had not been previously described for *Vitis* and are not included within the current *Vitis* TIGR collection. About 22% of these transcripts bear no resemblance to any known plant transcript and thus corroborate the need for this targeted EST collection. The added value of the presented EST collection lies in the afforded ability to carry out computational expression profiling, in order to identify genes/functions whose expression is altered in response to the dormancy-release treatment. Based on this analysis, it is suggested that oxidative stress, calcium signaling, intracellular vesicle trafficking and anaerobic mode of carbohydrate metabolism play a role in the regulation and execution of grape-bud dormancy release.

INTRODUCTION

The buds of temperate woody plants, including grapevine, undergo a dormancy cycle during the winter, which is induced by decreasing photoperiod and/or temperatures (Heide, 1974; Amling and Amling, 1980; Walser et al., 1981; Hummel et al., 1982; Ketchie, 1985; Scalaberilli and Couvillon, 1986; Fennell and Hoover, 1991; Rodriguez et al., 1994), and ends when plants have received adequate chilling temperatures and are exposed to favorable growing conditions (Samish, 1954; Brown et al., 1967; Saure, 1985; Dennis, 1986).

In warm-winter regions, prolonged dormancy is a major obstacle for commercial production of temperate fruits, including grapevine, which is widely distributed in subtropical regions (Shulman et al., 1983; Saure, 1985; George et al., 1986; Erez, 1987). At present, the control of dormancy release by the use of artificial dormancy-breaking compounds is needed to compensate for lack of natural chilling and is indispensable for maintaining economic production of table grape in these regions (Erez, 1985, 1987, 1995). However, the currently available effective compounds are both costly and entail a risk of bud damage due to their phytotoxicity (Erez, 1987, 1995; Or et al., 1999).

An understanding of the biological mechanisms involved in bud-dormancy release is crucial for the manipulation of bud-break timing. Although extensive studies have been performed on various physiological aspects of dormancy (Nitsch, 1957; Fuchigami et al., 1982; Saure, 1985; Dennis, 1986; Fuchigami and Nee, 1987; Powell, 1987; Martin, 1991; Lang, 1994; Borchert, 2000), a characterization of the complex network of biochemical and cellular processes responsible for the regulation and execution of bud-dormancy release has not yet been achieved (Seeley, 1994).

Application of hydrogen cyanamide (HC), which is the most useful dormancy-breaking agent for grape (Shulman et al., 1983; Erez, 1987, 1995; Henzell et al., 1991), provides a controlled system for the analysis of dormancy release in grape buds, with a relatively uniform response of the bud population. The controlled induction timing and the uniform response make this system an optimal tool for accurately defining the actual timing of induction of dormancy release, thereby enabling the detection of early changes following this induction (Or et al., 1999, 2000a, 2000b, 2002).

The mechanism by which HC exerts its dormancy-breaking effect is not clear, but it has been shown that both catalase expression and activity are inhibited in grape buds shortly after HC

application, leading to the accumulation of hydrogen peroxide (Nir et al., 1986; Shulman et al., 1986; Or et al., 2000b). We recently described GDBRPK, a transcript for a sucrose non-fermenting (SNF)-like protein kinase that is up-regulated following chemical induction of dormancy release by HC. We also described the simultaneous and remarkable transient induction of transcripts encoding pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) shortly after HC application. Based on these findings, we speculated that HC application leads to transient respiratory stress, which likely results in a temporary increase in the AMP-to-ATP ratio (Or et al., 2000b). Since AMP is known as a stress signal that is sensed by SNF-like kinases, we suggested that the SNF-like GDBRPK could serve as the sensor of this signal (Or et al., 2000b, 2002). However, the identification of such changes in expression patterns of single genes provides only partial clues to the possible involvement of specific metabolic pathways in the dormancy process.

To gain a broader perspective on the complex biochemical network responsible for the regulation and execution of the dormancy process, much more detailed insight is needed into the coordinated induction (or repression) of metabolic cassettes that act together during dormancy release in grape buds. An initial step in that direction is the application of genomic approaches to the characterization of alterations in gene expression following controlled endodormancy release. The large *Vitis* EST collection in the TIGR database and the *Vitis vinifera* Affymetrix microarray are important tools for grape genomics. Nevertheless, bud tissue in general, and the dormant bud in particular, are under-represented within these resources. Therefore, we generated an EST collection from grape buds that consists of randomly selected clones from four different cDNA libraries originated from young and mature buds at various developmental stages. In this work, we present a collection of 5516 consensus sequences, of which 59% were not formerly described for *Vitis* and are not included within the current *Vitis* TIGR collection. About 22% of these transcripts bear no resemblance to any known plant transcript, corroborating the need for this targeted EST collection.

An added benefit of the current collection lies in the conferred ability to compare EST frequencies between the different cDNA libraries. Computational expression profiling of the presented EST collection was implemented to identify genes/functions whose expression is correlated with particular points in bud development or induced in response to the dormancy-release treatment.

RESULTS

HC Mediates Controlled Induction of Dormancy Release

Detailed studies carried out in the Jordan Valley over several years showed that at the beginning of September, cv. Perlette buds are mostly non-dormant. Thereafter, endodormancy develops, reaching a maximum level at the beginning of November. This endodormancy is overcome at the beginning of January (Or et al., 2000a, b). Based on these findings, we usually perform controlled induction of dormancy release by HC in mid-December. At this stage, the bud population is still dormant, but it is close to the transition point from the endodormancy stage, in which bud growth is repressed by physiological factors inside the bud, to the ecodormancy stage, in which bud break is avoided because of environmental factors that do not support growth (Lang, 1987).

Correct timing of chemical induction is needed to allow controlled endodormancy release and yet avoid HC toxicity symptoms, which are dormancy-stage-dependent and often appear as the resistance to the chemical declines rapidly upon release from endodormancy (Shulman et al., 1983; Fuchigami and Nee, 1987; Erez, 1995).

To verify the success of the artificial induction and to provide bud materials presenting a relatively uniform response, bud-break response of single-bud cuttings under forcing conditions was analyzed following HC treatment: this is a common indicator used to describe depth of dormancy in grapevines (Emmerson and Powell, 1978; Shulman et al., 1983; Koussa et al., 1994; Lavee and May, 1997).

Under controlled conditions, HC induced around 80% bud break 24 days after its application, while buds on control vines showed only about 20% bud break (Fig. 1), suggesting powerful and uniform chemical induction of dormancy release.

Global Analyses of the Creation of Consensus Sequences (Unigenes)

An EST collection was generated from 7920 randomly selected clones originated from four different cDNA libraries (see Materials and Methods). Two groups of 2400 and 2352 clones were selected, respectively, from two libraries: one representing mature dormant buds

sampled at different time points following controlled endodormancy release by HC (lib. A) and the other their respective controls (lib. B).

A group of 1248 clones were selected from a library originated from mature buds sampled at the vineyard from mixed positions along the woody cane during the dormancy cycle (lib. C). Another group of 1920 clones originated from a library which was constructed from young buds sampled along the shoot from bud break to veraison (lib. D).

The clones were sequenced in both directions and thus the initial data set contained 15,840 sequences (representing 7920 clones), from which a subset of 14,001 high-quality sequences remained after the cleaning process. After processing, the clean data set was reduced to 9065 unigenes comprising 1979 contigs (6915 sequences) and 7086 singletons. These unigenes were termed Volcani Unigene List (VUL). The unigene lengths varied from 222 to 3489 bp (data not shown). The number of sequences per contig varied from 2 to 253 (Fig. 2).

A second assembly process which merged the original VUL with TIGR Unigene production produced 29,858 unigenes comprising 2445 contigs and 27,413 singletons. Removal of all the contigs and singletons that had no Volcani representative (see Sequence file S1 in Supplemental Materials), and corrections for clone mates (see Materials and Methods), reduced the number to 5516 unigenes: 2238 'merged contigs' (MC) and 3278 'merged singletons'. The merged singletons represented 330 Volcani contigs (VC) and 2948 Volcani singletons (VS). Based on these numbers, 59% of the unigenes produced during the second assembly represented new sequences not included in the current TIGR Unigene set.

The MC length ranged from 196 to 4090 bp with an average and median of 1163 and 1130 bp, respectively, while the length of the VC created in the first clustering cycle ranged from 222 to 2513 bp, with an average and median of 845 and 718 bp, respectively. The length of the TCs that were included in the MCs varied from 133 to 4090 bp, with an average length of 964 and median of 868 bp (see Fig. S1 in Supplemental Materials).

Functional Annotation of Merged Unigenes

The merged unigene set was annotated by similarity searches that were carried out locally against the NCBI NR, SwissProt and Trmbl databases. The similarity searches were performed with all merged unigene sequences, to gain maximum sequence information at the

annotation stage. These searches yielded 6028 hits to NR, 5918 hits to Trmbl and 3911 hits to SwissProt, all with E values below 1×10^{-5} . The final numbers of hits after the clone-mate correction (5516 unigenes) were 4304, 4229 and 2842 hits to NR, Trmbl and SwissProt, respectively, with E values below 1×10^{-5} .

Of 1212 unigenes that had no hits to NR, 160 were MCs, 190 were VCs (total of 501 sequences) and 862 were VSs. A detailed description of these unigenes is presented in Table I. TBLASTX similarity searches against the TAIR Arabidopsis unigene database revealed only 33 of these unknown sequences showing significant similarity to an Arabidopsis transcript of unknown function.

Differential Expression of Genes

The abundance of a certain transcript in an EST collection from a non-normalized cDNA library can be used to estimate its expression level in various tissues and under different treatments (Fei et al. 2004; Ma et al., 2004). Differential gene expression was computed based on the digital expression profile for library A versus B (i.e. HC vs. controls) and library C versus D (mature vs. young). Each gene was scored with an R value (Stekel et al., 2000) expressing the degree/significance of the difference in clone counts. Based on the validation results (see below), genes with an R value greater than 1.44 were considered differentially expressed. This analysis detected 30, 37, 57, and 36 genes that were up-regulated in libraries A, B, C and D, respectively (see Table S1 in Supplementary Material).

The digital expression profile identified remarkable differences in the abundance of clones for certain genes between the HC and control libraries. Genes were selected for validation of differential expression based on their in-silico expression profile and their potential ability to shed light on components of the dormancy-release mechanism. We validated the variation in expression following HC treatment for seven genes with increased expression and two genes with decreased expression (Table II). The expression profiles of all of the genes matched the prediction (Fig. 3).

Northern analyses revealed that apart from sucrose synthase and actin depolymerization factor, all genes showed increased transcript level as early as 12 h after treatment. Polyubiquitin and calmodulin-binding protein (CBP) showed maximal transcript level 1 day after treatment. Later, the transcript's level decreased; however, some still

remained, while it was absent in the respective control. The other five induced genes, namely Rab GTP-binding protein, actin depolymerization factor, sucrose synthase, glutathione S-transferase (GST) and thioredoxin h, showed a notable increase in transcript level after 1 day, which increased further and peaked after 2 days, and then declined slightly at 4 days. Expression level of the HC-induced genes was generally very low in the control buds at all time points. For both dehydrin and auxin-repressed protein, maximal reduction was evident 48 h after treatment.

Likewise, the gene expression profile was validated for selected genes showing differential expression between the mature, woody buds (lib. C) and the young, green buds (lib. D). Young and mature bud RNA mixes, each composed from buds taken at several time points, were used to examine expression levels for three genes. While dehydrin and disulfide oxidoreductase presented much higher transcript levels in mature buds, the chalcone synthase gene showed the opposite behavior (Fig. 4).

Functional Profile of Libraries Based on Gene Ontology

For each unigene in our collection that had a significant SwissProt hit, we assigned a GO classification. This assignment was limited since only 45, 52, 45 and 72% of the unigenes in libraries A, B, C and D, respectively, showed significant similarity to a SwissProt accession. In this context, it is interesting to note the relatively higher number of sequences in the 'young bud' library that were similar to SwissProt proteins. This probably reflects the fact that substantially more knowledge is available regarding green tissues as compared to the woody bud, which was the tissue used to construct the other libraries.

The number of clones for a given GO term was summarized and its percentage of the total number of clones in the relevant library calculated. Having said that our analysis may be biased due to the large fraction of genes that are currently out of analysis, and that functional assignment based on sequence similarity requires experimental verification, this nevertheless provides some insight into functions that are influenced by treatment or developmental stage.

The biologically relevant comparisons were between the HC-treatment library and its control (A vs. B: effect of treatment) and between the field-originated mature and young bud libraries (C vs. D: effect of developmental stage). These comparisons revealed significant

differences between the libraries for quite a few GO terms (see Table S2 in Supplementary Materials), of which a partial collection is presented here (Figs. 5, 6).

Under the main category of molecular function, the comparison between libraries A and B exposed significant differences in activities, such as those of ligase, two types of transferase, monooxygenase, hydrolase, anion transporter, cis-trans isomerase, RNA binding, polysaccharide binding and electrochemical potential-driven transporter (Fig. 5a). Under the main category of cellular component, there were significant differences in the ubiquitin ligase complex, ribonucleoprotein complex, β -galactosidase complex and plasma membrane terms (Fig. 5b). Within the biological processes GO, significant differences were detected in cell division, cell cycle and biopolymer metabolism terms (Fig. 5c).

A comparison between the libraries prepared from mature (lib. C) and young (lib. D) buds exposed significant differences in transferase, hydrolase, monooxygenase and ATPase activities under the molecular function main category (Fig. 6a). Among the cellular components (Fig. 6b), there were marked differences in the thylakoid term as well as in the ubiquitin ligase complex and ribonucleoprotein complex. Within the biological processes category, response to stress and response to biotic stimulus terms appeared to be significantly higher for library C (Fig. 6a), while signal transduction, carbon utilization, photosynthesis, regulation of metabolism, secondary metabolism, cell cycle and cell division were higher in library D.

To verify whether the quantitative difference between the compared libraries for a particular term stems from the induction of a single gene or represents a change in a wider set of functionally related genes, we analyzed the content of several terms presenting marked differences. The unigenes representing each analyzed GO term were listed and the number of clones for each of the unigenes summed separately for each of the libraries being compared (see Table S3 in Supplemental Materials).

This analysis showed that the difference in the ubiquitin ligase complex between libraries A and B (5A:28B) stems mainly from the existence of 15 clones coding for RING finger proteins in library B and only one clone in library A. Similarly, the difference in the monooxygenase category (6A:15B) stems mainly from a higher number of cytochrome p450 clones in library B compared to library A (4A:11B). The difference in the transferase activity of the acyl group (50A:22B) turned out to be due to a much higher number of stilbene synthase clones in library A compared to library B (38A:10B). On the other hand, for the

electrochemical potential-driven transport and RNA binding terms, no specific gene could account for the difference, which stems, rather, from the induction of a new set of genes due to the change in the developmental stage of the bud.

Similar comparison of libraries C and D showed that differences in the terms 'response to stress/response to biotic stimulus' are in large part due to a significantly higher number of heat-shock proteins (40/113 in lib. C compared to 12/116 in lib. D) and pathogenesis-related proteins (22/113 in lib. C compared to 5/116 in lib. D) in library C. On the other hand, the functional difference between the young and mature buds led to a marked difference, both quantitative and qualitative, in the signal transduction (9C:46D) and thylakoid (5C:44D) subcategories. For these terms, library D presents a much bigger variety of functions which are naturally related to active differentiation and growth.

DISCUSSION

Remarkable progress has been made recently in the public availability of data and tools for genomic analysis of *V. vinifera*, such as a wide *Vitis* EST collection in the TIGR database (Goes da Silva et al., 2005) and an Affymetrix microarray. However, bud tissue in general, and the mature dormant bud tissue in particular, are under-represented within these resources. Although a pioneering report on a private collection of mature grape-bud ESTs was previously published (Pacey-Miller et al., 2003), the collection reported here is the first public effort to generate a large-scale grape-bud EST collection that represents a wide range of developmental stages, from the very early stages of young bud development to bud maturation. Of special interest is the focus on the issue of controlled artificial bud-dormancy release, which allows the identification of events taking place early after induction of dormancy release. An identification of such events is crucial for characterizing the complex network of biochemical and cellular processes responsible for the regulation and execution of bud-dormancy release.

A collection of 15,840 bud-derived ESTs, representing 7920 clones and tagging 5515 unigenes, was established. The obtained EST sequences were of high quality, covering both 5' and 3' sequences of each of the clones, from which only about 5% were discharged as being of low quality or low complexity. Although the libraries were not normalized, most of the sequences present in the unigene set were single or low-copy genes. Among these unigenes,

59% (3278) had not been formerly described for *Vitis* and were not included in the current *Vitis* TIGR collection. In addition, 1212 unigenes (over 20% of the unigene set) showed no similarity to any known sequences in the NCBI 'NR' database. Out of these 1212 unigenes, only 160 were formerly described for *Vitis* and were included in TIGR prior to the current study. While it is possible that a few of the remaining 1052 unigenes represent untranslated regions or are too short to have domains that would present significant matches with other proteins, the average and median values of sequence length for this group of genes suggest that most should include coding sequences, and therefore it is reasonable at present to consider them novel grapevine genes. Taken together, our results support our main premise regarding the present need for a targeted bud EST collection and an expansion of the overall collection of *Vitis* ESTs.

The main goal of this sequencing project was to provide a genomic foundation for an elucidation of the complex network of biochemical and cellular processes responsible for the regulation and execution of bud-dormancy release. Therefore, in addition to the novel genes discovered, the added value of the current collection lies in its potential to enable comparative analyses of EST frequencies among the different cDNA libraries.

The list of unigenes showing a significant difference in redundancy between libraries contained a mixture of genes with both known and unknown functions. In addition to the unigenes that matched only unknown or hypothetical proteins, our collection includes a large number of unigenes that currently show no significant similarity to any known gene. Although the genes of unknown function provide a valuable resource for new insight into the biology of dormancy release, their functional analysis was beyond the scope of this study. Putting these groups aside, we concentrated our validation effort on annotated unigenes that might shed light on the process of dormancy release.

In general, the balanced selection of an array of unigenes, presenting both increased and decreased transcript levels within a given pair of compared libraries, and the validation of the expected pattern for all the selected unigenes, proved the reliability of the entire set of results, and allowed us to use clone counts as a valid measure of differential expression of a unigene between the compared libraries.

Differences between the young, green buds and mature, woody buds during the dormancy cycle were validated for three unigenes that were present selectively in either mature or young bud libraries. The transcript level of chalcone synthase, catalyzing the first

committed step in the flavonoid-biosynthesis pathway, was high in the young buds and undetectable in the mature ones. On the other hand, dehydrin transcripts were highly expressed in the dormant buds and undetectable in the young buds. In addition, it was apparent that dehydrin expression is shut down during the controlled dormancy release induced by HC. Dehydrins are proteins that are normally synthesized in situations of cellular dehydration, such as occurs during certain developmental stages or in response to certain environmental stimuli. Dehydrins are present in maturing seeds and mature buds exposed to chilling and have been shown to disappear when growth is renewed (Lang, 1994; Muthalif and Rowland, 1994; Salzman et al., 1996; Faust et al., 1997; Welling et al., 1997, 2002; Rinne et al., 1999). The reduction in transcription level observed in the current study is supportive of the effect of HC on growth resumption as early as 48 h after its application, and suggests that reduction of dehydrin transcript level is controlled by dormancy-status regulators that are not dependent on chilling temperature, since HC-induced dormancy release was carried out at room temperature. Nevertheless, the cascades of chilling and HC signals may share putative sensors.

A second gene that was up-regulated in mature buds as compared to young ones was annotated as a "putative protein presenting disulfide oxidoreductase activity." A large number of proteins containing cysteines with free thiols can be modified by the formation of internal disulfides or mixed disulfides with low-molecular-mass thiols, usually glutathione. In the past, protein glutathionylation and disulfide formation were viewed as repair mechanisms of protein damage in the context of defense against oxidative stress. However, there is now growing interest in oxidoreduction of protein thiols/disulfides as a regulatory mechanism (Ghezzi, 2005). Apart from this unigene which is induced in naturally matured dormant buds, the comparison of HC-treated and control bud libraries revealed the significant induction of another, more characterized member of the disulfide oxidoreductase family, thioredoxin h. Thioredoxins play many roles in the cell, including redox regulation of target enzymes and transcription factors. They also serve as hydrogen donors to peroxiredoxins, the recently discovered heme-free peroxidases that reduce hydroperoxides to water (Jacquot et al., 2002; Sweetlove et al., 2002; Marchand et al., 2004; Serrato et al., 2004). Accordingly, induction of thioredoxin transcript level has been reported in response to oxidative stress (Serrato and Cejudo, 2003; Laloi et al., 2004; Rey et al., 2005) and thioredoxin h, which is usually

localized to the cytosol, was recently proven to reside in the mitochondria and regulate the activity of the alternative oxidase (Gelhay et al., 2004).

Previous studies have revealed that HC inhibits catalase activity in both animal (Kitson and Crow, 1979; DeMaster et al., 1982; Shirota et al., 1984) and plant (Amberger, 1984) systems, including grape (Nir et al., 1986). We recently showed that catalase transcription is inhibited in grape buds shortly after HC application (Or et al., 2002). Inhibition of catalase activity has led to oxidative stress in several systems, due to increased content of hydrogen peroxide (Godon et al., 1998; Prasad, 1996; Dat et al., 1998).

Based on the above, and on the induction of the anaerobic machinery following HC application (Or et al., 2000b), we previously speculated that HC application leads to the development of oxidative stress (Or et al., 2000b). The induction of thioredoxin h expression in response to HC application supports this hypothesis.

Apart from catalase, there is a range of peroxidases that reduce hydrogen peroxide to water and are induced in response to oxidative stress. Among them are ascorbate peroxidase, glutathione peroxidase and peroxiredoxins. Electrons for the latter reactions are supplied by NADPH via ascorbate, glutathione or thioredoxin (Noctor and Foyer, 1998). Accordingly, further support for the above raised hypothesis comes from the major induction of GST transcript level following HC application. Among other functions, GSTs function as glutathione peroxidases, reducing the organic hydroperoxides produced during oxidative stress. They also function as dehydroascorbate reductases (DHARs), reducing dehydroascorbate to ascorbic acid to allow proper function of the ascorbate-glutathione cycle, a major player in the antioxidant defense system (Edwards and Dixon, 2005). Expression of GST1 transcript in *Arabidopsis* is induced by oxidative stress (Reuber et al., 1998; Kliebenstein et al., 1999) and elevated mRNA levels of GST are widely used as early markers of biotic and abiotic stress in this model plant (Grant et al., 2000; Rate and Greenberg, 2001). Recently, import of the major cytosolic GSTA4-4 into mitochondria was demonstrated in mouse cells following oxidative stress, and a role for this enzyme in the removal of lipid-peroxidation products has been established (Raza et al., 2002). Available evidence in mammalian systems strongly suggests that GSTs play an important physiological role in the regulation of cellular signaling processes (Awasthi et al., 2005).

Accumulating evidence suggests that dormancy-breaking agents, such as high temperature, chemicals and chilling, may all lead to the development of oxidative stress

(Prasad, 1996; Dat et al., 1998). It is now widely accepted that oxidative stress may be involved in signal transduction (Conrath et al., 1997; Foyer et al., 1997; Anderson et al., 1998). Based on our findings so far, we suggest that oxidative stress develops during dormancy release and may be involved in the signaling cascade leading to the dormancy release. Our data are partially supported by those published by Pacey-Miller et al. (2003) who showed induction of glutathione reductase and glutathione peroxidase transcript levels 3 to 4 weeks before natural bud burst. Based on our vast experience with induced dormancy release of dormant grape buds, it takes about 4 weeks under spring field conditions from controlled induction of dormancy release (by HC or heat shock) to actual bud break (Or et al., 1999, 2000a and unpublished data). Therefore, the induction reported by Pacey-Miller et al. (2003) probably represents events occurring during the dormancy-release stage. In this context, it is important to mention that their study presented only one or two time points during dormancy release, taken 7 days apart, whereas we have indications that changes are transient and occur within 2 to 3 days of controlled artificial induction (Or et al., 1999, 2000b, unpublished data). Combined with the lack of a physiological characterization of the buds' dormancy status at the sampling time points, the preliminary data presented by Pacey-Miller et al. (2003) requires a much more detailed follow-up.

In addition to the induction of oxidative stress-related genes, there were some other genes that were clearly induced in the buds when challenged with HC. Among these were Rab GTP-binding protein, actin depolymerization factor (ADF), ADP-ribosylation factor (ARF), calmodulin binding protein (CBP), sucrose synthase and polyubiquitin.

Similar to the aforescribed, analysis of the impact of oxidative stress on the proteome of plant mitochondria revealed the combined induction of disulfide isomerase, peroxiredoxin, GST, ADF and the endoplasmic reticulum-based Ca^{2+} -sequestering protein calreticulin (Sweetlove et al., 2002). Differential expression of ADF has also been associated with cold hardiness in wheat, a response that increases dehydration tolerance (Ouellet et al., 2001).

The combined induction of ADF and genes related to the function of the secretory pathway, namely Rab GTP-binding protein and ARF, suggests a central role for the intracellular vesicle trafficking system in dormancy release. Both ARF, and Rab and Rho GTPases are classes of small GTPases which are active in the secretory pathway and have been implicated in regulating cytoskeletal dynamics and molecular motor function within this

pathway (Chen et al., 2005). The functional connection between the three unigenes induced in the current study was formerly demonstrated in the Golgi apparatus membrane, where the Rho GTPase activates actin polymerization following recruitment of a complex between the vesicle-coat protein and a Rho GTPase by ARF1 (Chen et al., 2005).

Involvement of small GTPases in the modulation of hydrogen-peroxide production has been reported (Mazel et al., 2004). Induction of a Rab GTPase, a vesicle traffic-regulating protein, was evident following treatment of *Arabidopsis* intact leaves with superoxide. Overexpressors of the gene showed increased tolerance to salt and osmotic stresses and reduced the accumulation of reactive oxygen species during salt stress (Mazel et al., 2004), suggesting that such activities may be required during oxidative stress. Rho-related GTPases regulate actin dynamics and modulate hydroperoxide production during polar cell growth (Molendijk et al., 2004).

CBPs, the expression of which was markedly induced following HC application, interact with calmodulin in a Ca^{2+} -dependent manner. Although the function of the CBP is unknown, its marked induction following HC application suggests Ca^{2+} -signaling's involvement in grape-bud dormancy release. Calcium is a prominent second messenger in signal-transduction cascades in plants, involved in a wide variety of growth and developmental processes, including plant responses to various environmental stimuli (Hepler and Wayne, 1985; Bush, 1995; Trewavas, 1997). Recently, oxidative stress has been shown to induce Ca^{2+} -signaling in plant cells (Pei et al., 2000). Interestingly, the interaction between calmodulin, ARF, and Rho-family GTPases is involved in regulation of the actin cytoskeleton, receptor signaling and vesicle trafficking in mammalian systems (Takahashi et al., 1996; Jenkins and Frohman, 2005).

Another gene that was clearly induced in the buds when challenged with HC was annotated as sucrose synthase, catalyzing sucrose cleavage to fructose and UDP glucose. The functional significance of sucrose synthase is particularly important under low oxygen conditions and its requirement for survival under such conditions was recently proven using sucrose synthase-deficient mutants (Subbaiah and Sachs, 2003; Koch, 2004). It belongs to a small group of glycolysis and fermentation-related enzymes which are up-regulated under such conditions, and apparently function to allow limited energy production in the face of limited oxygen supply (Subbaiah and Sachs, 2003). Our data, presenting coordinated induction of sucrose synthase, PDC and ADH (Or et al., 2000b) following HC application

suggest that HC affects the tissue in a way that mimics anoxic conditions and suggest the mitochondria as a potential sensing center for the HC signal. Sucrose-synthase up-regulation is also evident in response to cold shock, water deprivation and salt stress (Marana et al., 1990; Baud et al., 2004; Gu et al., 2004). In the last case, sucrose-synthase up-regulation is accompanied by calmodulin up-regulation (Gu et al., 2004), like in this work. Cytosolic Ca^{2+} has been proven to be a transducer of low oxygen signals in maize seedlings, leading to changes in sucrose synthase gene expression as well as changes in other genes (Subbaiah and Sachs, 2003). Thus cytosolic Ca^{2+} might be a transducer of the HC signal as well.

A potential role for sucrose synthase in dormancy release may be inferred from findings connecting sugar-sensing systems and sucrose synthase to regulation of plant-hormone biosynthesis and sensing, and to meristem development. In this context, it is interesting to note that the sucrose synthase gene is one of the first genes induced as leaf primordia differentiate from the apical meristem (Pien et al., 2001; Koch, 2004).

This enzyme is typically soluble in the cytoplasm and can contribute readily to an ATP-conserving path of respiration. However, recent work has indicated a broad spectrum of apparently transient associations of sucrose synthase with membranes and/or actin. Such associations may mediate a number of special functions at the plasma membrane, tonoplast and Golgi sites, and that with actin could facilitate metabolic shuttling (Koch, 2004). Cellular sugar status, developmental stage and phosphorylation appear to influence the shift of sucrose synthase from cytoplasmic to membrane-bound forms (Winter et al., 1998; Winter and Huber, 2000; Koch, 2004).

We assigned GO classifications to each unigene that had a significant SwissProt hit. The number of clones for a given GO term was summarized and its percentage of the total number of clones in the relevant library calculated. We assume that the number of non-annotated clones, which were not included in the current analysis, reflects additional categories, presently lacking a GO classification, rather than additional clones from existing GO categories. Based on this assumption, we believe that a quantitative comparison of a GO term's presence in the libraries provides insight into functions that are influenced by treatment or developmental stage.

To verify whether the quantitative difference between the compared libraries for a particular GO term stems from the induction of a single gene, or represents a more global change in function, we analyzed the content of several GO terms that exhibited marked

differences. When the treatment and control libraries were compared, it was clear that changes in several GO terms reflected mainly a change in the expression of a single gene. This is indicative of the dramatic effect of the treatment on the expression of certain genes that might be key genes, as discussed earlier.

On the other hand, the "functional distance" between the young and mature buds led to both quantitative and qualitative differences within the clone populations in each of the analyzed categories. In all of them, the young bud library (library D) presented a much larger variety of functions which are naturally related to active differentiation and growth.

Although it is well documented that mature buds are biochemically active, the kind of data presented here clearly proves that young and mature buds carry a different degree, as well as a different repertoire of biochemical activities. It also shows that the young, green buds function as active photosynthetic tissue.

The EST collection generated in this study provides an invaluable information resource for characterizing genes involved in bud development in general, and dormancy release in particular. We expect that microarray analyses targeted specifically to the effect of HC application, as well as alternative artificial signals, will provide a more complete global understanding of dormancy release in grapevine. We therefore intend to carry out further expression studies with these genes using cDNA microarray, to provide new insight into the network of biochemical and cellular processes responsible for the regulation and execution of bud-dormancy release.

MATERIALS AND METHODS

Plant Material

The experiments were conducted with mature, cordon-trained grapevines (*Vitis vinifera* cv. Perlette and cv. Superior) from commercial vineyards. All plants were subjected to the cultural practices commonly used in such vineyards. Mature buds were collected from canes originated from a cv. Perlette vineyard in the central Jordan Valley. Young buds were collected from green shoots of cv. Superior vines from a commercial vineyard located in the plain of Judea.

Collection of mature buds

Natural dormancy cycle. From November to January, vines at a cv. Perlette vineyard in the Jordan Valley were pruned at 2-week intervals to three-node spurs and the detached canes, each containing nine buds (in positions 4-12) were transferred to the lab. Canes were cut into single-node cuttings that were randomly mixed. Ten groups of 10 single-node cuttings were prepared on each collection date, placed in water and then forced for 21 days at 23°C under a 12/12 h photoperiod. The bud-break percentages after 21 days were used to express the dormancy depth of the vines on the collection date as previously described (Or et al., 2000b). Bud break was defined as the stage at which green tissue is visible beneath the bud scales. Buds were harvested from the detached canes from each collection date and were immediately transferred to liquid nitrogen and stored at -80°C.

Chemical induction of dormancy release. Dormancy-breaking treatments were applied in mid-December. Vines were pruned as already described and the detached canes were divided into two groups. Canes of one group were sprayed with 5% Dormex, a commercial formulation containing 49% HC, and those of the other group were sprayed with water and served as controls. Both Dormex and water were sprayed with the addition of a 0.02% (v/v) Triton X-100 wetting agent. Sprayed canes were cut into single-node cuttings that were randomly mixed and placed in water in a growth chamber at 23°C under neutral 12/12 h light/dark photoperiod. Buds were harvested from both HC-treated and control cuttings at selected time points after HC application (3, 6, 12 and 24 h, 2 d and 4 d), and were immediately transferred to liquid nitrogen and stored at -80°C.

Ten groups of 10 single-node cuttings were prepared from control and HC-treated canes and then forced, under the conditions described above. Bud-break percentages were calculated and used to assess the effect of the treatment on dormancy release.

Collection of young buds

Green buds were harvested from young shoots of the already described *Vitis vinifera* cv. Superior vines. Collection was started in March, 4 weeks after bud burst, and continued at weekly intervals throughout bud development until veraison, in mid-June.

Extraction of RNA and Purification of Poly(A)⁺ RNA

RNA was extracted from frozen bud samples using a modified CTAB-LiCl protocol (Chang et al., 1993). PolyA-mRNA was purified from total RNA using Promega's PolyATtract mRNA Isolation System according to the manufacturer's instructions.

Construction of cDNA Libraries

Four different bud cDNA libraries were constructed. Two libraries were constructed from mature buds following the aforescribed chemical induction of dormancy release. Equal quantities of total RNA from buds sampled at each time point following HC treatment were mixed. The mixed RNA sample was used for PolyA-mRNA preparation and cDNA was synthesized using SMART-PCR cDNA synthesis technology (ClonTech). A similar procedure was performed for the control buds. The cDNA was then used to create two separate cDNA libraries by directional cloning into TripleEx2 vector (Clontech). The HC library was termed library A and the control library was termed library B.

A natural dormancy cycle bud library was constructed from RNA originated from the buds collected from mature canes at 2-week intervals during dormancy and dormancy release. This library was termed library C. A young cv. Superior bud library was constructed from RNA originated from the young buds collected from green shoots on a weekly basis from March to June. This library was termed library D. For both libraries, equal quantities of total RNA from each collection date were mixed and used for PolyA-mRNA preparation, which was directionally cloned into a ZapII vector (Stratagene) following the manufacturer's instructions.

Selection of ESTs

Individual clones were randomly selected from the libraries after in-vivo mass excision according to the manufacturer's instructions. The resulting plasmids were pBluescript II (from the ZapII vector) and pTripleEx2 (from the TripleEx2 vector), which were then propagated in the *E. coli* strains SOLR and BM25, respectively. Cell-culture stocks were created (in 96-well plates) for 1248 clones from the natural dormancy cycle mature bud library, 1920 clones from the young bud library, 2400 clones from the HC library and 2352

clones from the control library. The total collection from all four libraries amounted to 7920 clones.

Sequence Processing and Creation of Consensus Sequences (Unigenes)

Raw sequences (15,840 ESTs), resulting from the sequencing of 7920 clones from both their 5' and 3' ends, were cleaned of vector sequences and low-quality regions using SeqClean (<http://www.tigr.org/tdb/tgi/software/>). Resulting sequences that were shorter than 100 bp or that consisted mostly of low-complexity successions were discarded. The resulting 14,001 high-quality sequences were analyzed with TGI Clustering (TGICL), a software system for the rapid clustering of large EST data sets (<http://www.tigr.org/tdb/tgi/software/>). The parameters used for clustering allowed the grouping of sequences sharing a minimum of 95% identity over at least 40 nucleotides with less than 20 bases of mismatched sequence at either end (Quackenbush et al., 2000). Initially, the sequences were searched for repeats using the Arabidopsis section of Repeatmasker Repbase libraries from 23/05/2005 (<http://www.repeatmasker.org/>). However, the grapevine ESTs did not show significant similarity to these repeats, and the repeat masking step of the unigene creation was omitted.

The resulting consensus sequences (unigenes) were named Volcani Unigene List (VUL). A second cycle of unigene creation was performed using the VUL and TIGR Gene Indices (tentative consensus, TCs) of *Vitis vinifera* (Release 4.0, Sep 21, 2004 ftp://ftp.tigr.org/pub/data/tgi/Vitis_vinifera/, 23,871 unigenes: 13,571 TCs and 10,300 singletons) as input. This process created a set of merged contigs constructed from VUL and TIGR TCs or singletons. After the merging step, we omitted contigs and singletons that contained only TIGR sequences and kept merged contigs that contained at least one VUL sequence. These unigenes were termed 'merged unigenes'.

Listing of Contigs and Singletons

To accurately estimate the number of unique clones, the 5' and 3' ESTs of each clone (termed "clone mates") were identified and the following corrections were implemented on the merged unigene list: in cases where one clone mate was part of a contig, while the other remained a singleton, the singleton was removed from the singleton list. When a contig was

comprised of only two clone mates, it was transferred from the contig list to the singleton list. When clone mates appeared as two different singletons, we omitted one of them from the singleton count. For 115 clones, clone mates were split into two contigs (2.7%): although this suggests that the two contigs belong to the same gene, we did not merge them.

Functional Annotation

Merged unigenes were annotated based on sequence similarity using BLASTX against the NCBI non-redundant (NR) database (<http://www.ncbi.nlm.nih.gov/Ftp/index.html>) and against SwissProt and Trmbl

(ftp://ftp.ebi.ac.uk/pub/databases/uniprot/current_release/knowledgebase/complete/).

Only significant BLAST results ($E \text{ value} < 1 \times 10^{-5}$) were considered (Lopez et al., 2004). For each of the merged unigenes, we used as an annotation the description of the target protein that was associated with the lowest E value.

Digital Expression Data for Grape Contigs

We present the expression levels as clone counts in a profile matrix that consists of 5516 rows for merged unigenes, including singletons, over four columns representing the cDNA libraries. Each cell of the matrix represents the number of clones observed for the corresponding unigene in the analyzed library. Significance of differential expression was estimated by assigning R values to the unigenes (Stekel et al., 2000). Threshold R values were chosen according to the validation results: all genes with R values equal to or greater than the R value of a positively evaluated gene were considered differentially expressed.

Northern Analysis

Total RNA from grape buds was denatured, fractionated by formaldehyde/agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N⁺, Amersham) according to Sambrook et al. (1989). Hybridization was performed with ³²P-labeled probes. The probes were amplified by PCR from the clones using insert screening primers (LDF and LDR) according to the manufacturer's instructions (Clontech). Probe was labeled by the random

primer method (Sambrook et al., 1989). Hybridization buffer containing 5X SSC and 10X Denhardt's Solution was prepared according to Sambrook et al. (1989). The hybridization temperature was 60°C. The membranes were washed twice with 2X SSC/0.1% (w/v) SDS for 20 min at room temperature, followed by a 15-min wash with 1X SSC/0.1% SDS and a 30 min wash with 0.1X SSC/0.1% SDS, both at 65°C.

Expression Profile of Clones Based on Gene Ontology

Expression profiles were performed for each library based on gene ontology (GO) categories. For each unigene with a significant SwissProt hit, the accession of the most significant hit was selected and assigned a GO classification using the Fatigo hierarchy (<http://www.fatigo.org/>). Level 4 GO classifications were chosen for each of the main ontologies (biological process, molecular function and cellular component). Digital GO category expression profile matrices were created in which the rows consisted of GO categories and the columns of the four libraries. Each cell of the matrix represents the number of clones from a certain library that appears in a specific GO category. Clone counts were compared for library A versus B and C versus D. Statistical analysis of the results was performed using chi-square tests of independence for each of the categories. A significant difference in clone counts was defined as $p(\chi^2) < 0.05$.

Supplemental Materials

Sequence file S1. Sequences of merged unigenes. Sequences are given in Fasta format.

Fig. S1. Comparison of length characteristics of merged contigs (MC), Volcani contigs (VC), and TIGR TCs. (A) Min/max length; (B) Average and median length.

Table S1. Digital expression profiles of the unigenes in the compared cDNA libraries. A and B represent HC and Control cDNA libraries. C and D represent mature and young bud libraries. Expression levels are presented as clone counts in a profile matrix that consists of rows representing the merged unigenes, and columns representing the cDNA libraries. Significance of differential expression was estimated by assigning R values to the unigenes.

Table S2. Matrices of GO category digital expression profile. The rows consist of GO categories and the columns of the four libraries. Each cell in the matrix represents the number of clones from a certain library that appears in a specific GO category. Clone counts were compared for library A versus B and C versus D. Statistical analysis of the results was performed using chi-square tests of independence for each of the categories. A significant difference in clone counts was defined as $p(\chi^2) < 0.05$.

Table S3. Analysis of clone composition of several GO terms presenting marked differences between the compared libraries. The unigenes representing each analyzed GO term were listed and the number of clones for each of the unigenes summed separately for each of the libraries being compared.

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FIGURE LEGENDS

Figure 1. Chemical induction of bud-dormancy release. Bud break of single-bud cuttings were induced by hydrogen cyanamide (HC) and cuttings were placed under forcing conditions in a growth chamber. Bars above the values represent the standard deviation of 10 repeats. The values are the averages of 10 replications.

Figure 2. Distribution of ESTs within Volcani contigs (VC)

Figure 3. Effect of HC application on transcript level of selected ESTs in grape buds.

Northern-blot analyses were conducted with equal quantities of total RNA (17 µg) extracted from HC-treated and control buds which were placed under forcing conditions in a growth chamber and sampled 12, 24, 48 and 96 h after treatment. RNA blots were probed with radiolabeled PCR products amplified from clones representing the following ESTs: dehydrin (TC45479); auxin-repressed protein (Aux DP; TC 38370); Rab GTP-binding protein (Rab GTP; TC 38504); actin depolymerization factor (Actin DPF; TC 25247); thioredoxin h (ThioR; TC 25169); glutathione S-transferase (GST; NP864091); sucrose synthase (Suc Syn; TC 31786); polyubiquitin 5 (Poly Ubiq; TC 38292); calmodulin-binding protein (CBP; TC 34227).

Figure 4. Transcript level of selected ESTs in young and mature grape buds. Northern analyses were conducted as described in Fig. 3 with equal quantities of total RNA mixes (17 µg), prepared separately from total RNA extracted from young and mature buds sampled at several time points during development. Radiolabeled probes were prepared from clones representing dehydrin (TC45479), disulfide oxidoreductase (OxRed ; TC45326), and chalcone synthase (ChSyn; TC45205).

Figure 5. Functional expression profile of clones based on GO main category. The number of clones for the indicated GO terms was summarized within each library and its percentage of the total number of clones in the relevant library was calculated. (a) molecular function; (b) cellular component; (c) biological process. A and B stand for HC and Control libraries, respectively. MF1---transferase activity, transferring alkyl or aryl groups; MF2-

--transferase activity, transferring acyl groups; MF3---RNA binding; MF4---ligase activity, forming carbon-nitrogen bonds; MF5---hydrolase activity, acting on glycosyl bonds; MF6---anion transporter activity; MF7---monooxygenase activity; MF8---electrochemical potential-driven transporter activity; MF9---cis-trans isomerase activity; MF10---polysaccharide binding; CC1---ribonucleoprotein complex; CC2---ribosome; CC3---plasma membrane; CC4---ubiquitin ligase complex; CC5---beta-galactosidase complex; BP1---biopolymer metabolism; BP2---cell cycle; BP3---cell division.

Figure 6. Functional expression profile of clones based on GO main category. The number of clones for the indicated GO terms was summarized within each library and its percentage of the total number of clones in the relevant library was calculated. (a) molecular function; (b) cellular component; (c) biological process. C and D stand for mature and young bud libraries, respectively. MF1---hydrolase activity, acting on glycosyl bonds; MF2---ATPase activity, coupled to transmembrane movement of ions; MF3---monooxygenase activity; MF4---transferase activity, transferring acyl groups; CC1---ribosome; CC2---thylakoid; CC3---ubiquitin ligase complex; CC4---ribonucleoprotein complex; BP1---response to stress; BP2---response to biotic stimulus; BP3---regulation of cellular physiological process; BP4---regulation of metabolism; BP5---cell cycle; BP6---signal transduction; BP7---secondary metabolism; BP8---cell division; BP9---photosynthesis, light reaction; BP10---carbon utilization. signal transduction; BP7---secondary metabolism; BP8---cell division; BP9---photosynthesis, light reaction; BP10---carbon utilization.

Table I. Characteristics of the unigenes that had no hits in the NCBI nr database

	Number of unigenes	Length range (bp)	Average (bp)	Median (bp)
Merged contigs	160	196 - 2013	855.7	752
Volcani contigs	190	251-1789	640.5	507.5
Singletons	862	125 – 3489	1186.8	1211

Table II. Digital expression profiles of selected genes across the different cDNA libraries. A, B, C and D represent cDNA libraries from HC-treated buds and their respective controls, mature buds, and young buds, respectively.

cDNA library		A		B		C		D	
Gene name	Unigene	Clone count	% of clones in lib.	Clone count	% of clones in lib.	Clone count	% of clones in lib.	Clone count	% of clones in lib.
Glutathione S-transferase	VC_grape_2976	6	0.25	1	0.04	0	0	0	0
Thioredoxin h	VC_grape_1022	34	1.42	13	0.55	0	0	0	0
Calmodulin binding protein	VC_grape_1213	4	0.17	0	0	1	0.08	0	0
Polyubiquitin 5	VC_grape_1199	3	0.13	0	0	0	0	0	0
Rab GTP-binding	VC_grape_1157	3	0.13	0	0	0	0	1	0.05
Actin depolymerization factor	VC_grape_1178	5	0.21	0	0	0	0	0	0
Sucrose synthase	VC_grape_1097	3	0.13	0	0	0	0	3	0.16
Dehydrin	VC_grape_1004	8	0.33	19	0.81	29	2.32	2	0.10
Auxin-repressed protein	VC_grape_1106	0	0	3	0.13	1	0.08	2	0.10
Chalcone synthase	VC_grape_1075	1	0.04	0	0	0	0	9	0.47
Oxidoreductase family protein	VC_grape_1012	2	0.08	1	0.07	41	3.29	0	0

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6