Characterization and manipulation of primary components potentially involved in ABA-mediated repression of grape bud dormancy release and in its removal

Or, E. Agricultural Research Organization
Galbraith, D.W. The University of Arizona AZ
Ophir, R. Agricultural Research Organization
Aharoni, A. Weizmann Institute of Science

Project award year: 2013
Three year research project
Abstract

We previously proposed a model for the cascade induced by artificial stimuli of grape bud dormancy release, which assigned central importance to the removal of ABA-mediated repression of meristem activity. Being the last identified link in the chain of events that induce dormancy release, removal of ABA-mediated repression of dormancy release was the central theme of the current study.

In hypothesis-driven mode, we focused on a selected core component of ABA metabolism, a bud-expressed ABA8ox, which is regulated by both HC and the natural dormancy cycle. We produced antibodies, and tracked changes in the enzyme level across the natural dormancy cycle and in response to HC, characterized its response to other dormancy release stimuli, and provided evidence that it is functional in vivo, and that over expression of this gene in grapevines enhanced bud dormancy release. We similarly characterized the potential involvement of selected components of ABA signaling, two ABRE genes which are down regulated by HC. Overexpression of these genes had an opposite effect, leading to a delay in bud break.

In discovery mode (1) we profiled the transcriptomes of HC, ABA-HC, and HC treated buds and compared these to the control, (2) we profiled bud transcriptome modulation throughout the dormancy cycle, and (3) we profiled the levels of primary metabolites as in (1) and (2). Based on the data that emerged, we further studied the potential involvement of sugar metabolism, gibberellin (GA) metabolism and Jasmonic Acid (JA) metabolism on dormancy release.

Our data suggest that (1) alterations in trehalose 6 phosphate and ABA levels may regulate dormancy by modulation of SnRK1 activity, (2) GA inhibits activation of the meristem, probably via antagonism to the effects of cytokinin, but enhances primordial growth metabolism, and (3) JA inhibits dormancy release, its effect depending on the dormancy status. Together, it appears that sugar signaling and hormone crosstalk are key components of this complicated developmental process, and may serve as specific targets for manipulation. The reader is encouraged to visit the detailed scientific report attached in order to assess our study in depth.
Summary Sheet

Publication Summary

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Training Summary

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Contribution of the collaboration

The research team was constructed due to its synergism potential, based on the diverse expertise and the productive records of Drs. Galbraith, Ophir, and Or, in a previously funded BARD grant. Together, the team has knowledge in bud physiology, molecular experience with the experimental systems, and grapevine transformation abilities (Or), excellent records in adopting and developing cutting-edge genomic and metabolomic technologies (Galbraith and Aharoni), and advanced bioinformatics expertise (Ophir). Each of these expertise was essential to carry out the study as detailed in the detailed scientific report attached. Specifically Galbraith, Ophir and Or were involved in identifying targets via comparative transcriptome profiling, while Aharoni and Or were involved in identifying targets of ABA in dormant grape buds via comparative non-targeted metabolite profiling. The Or lab carried the majority of the hypothesis driven part of the study, characterize and manipulating core components of ABA metabolism and signaling.
Achievements

In warm winter regions, where the table grape industry preferentially is located, artificial induction of bud dormancy release is mandatory for coordinate, early production of economical grape yields. The single effective artificial stimulus available for commercial use in vineyards is hydrogen cyanamide (HC). Unfortunately, the ability of HC to induce respiratory stress, which initiates a biochemical cascade that leads to effective dormancy release, is also responsible for its toxicity, both to the vines and to the environment. These phytotoxic effects, which results in irregular ripening and significant yield losses, coupled with new regulations that ban the use of HC in the deciduous tree industry from 2014, create an urgent need for the development of safe alternatives for artificial induction of bud dormancy release. Despite their high potential to serve as effective stimuli, searching for new chemical inducers of respiratory stress represents but a short-term vision, in that conservation of mitochondrial function across eukaryotes predicts generally toxic effects which would result in future ban of their use. A much better strategy is to search for targets which are affected by the induction of respiratory stress and that regulate bud dormancy release, since manipulation of these targets has a much better probability to be plant-specific and harmless to the environment. In the current study, we explored for such targets. Our results suggest that natural agents that affect ABA levels within the buds may become a viable means for controlled dormancy release. This could already be translated into a screen for such agents, and in initiating this process we believe we already have identified a potential candidate. Our results also suggest that genome editing of bud specific/oriented regulators of ABA synthesis/metabolism will be a viable means for control of dormancy release worldwide. This is another practical avenue that could be immediately applied and tested. Needless to say, the study revealed a much wider array of targets that can be further investigated for their practical benefits. To learn more, the reader is invited to examine the detailed report.
Changes made to the original research plan

We were able to produce antibodies against NCED, follow the enzyme level and prove its function in vivo, but we failed to produce over-expressing transgenic lines in time for functional testing during the period of grant support. We have repeated this experimental work, and now have produced young plantlets, and will perform functional tests once the vines are at the appropriate developmental stage.

Since we proved ABA8ox functionality in vivo, we skipped the in vitro tests which we had originally proposed.

We added profiling of the bud transcriptome throughout the dormancy cycle, which were not in the initial working plan.

Following up the profiling results, we added in depth studies of several candidate metabolic pathways (GA, JA, Sugars)
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<td>Abscisic acid (ABA) regulates grape bud dormancy, and dormancy release stimuli may act through modification of ABA metabolism.</td>
<td><em>J Exp Bot</em></td>
<td>66:1527-42 2015</td>
<td>IS only</td>
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<td>Sharabi-Schwager M, Or E, Ophir R</td>
<td>ctsGE – clustering subgroups of expression data</td>
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<td>: 2017</td>
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BARD Annual Scientific Report

Cover Page

Bard Project Number: IS-4639-13

Title: Characterization and manipulation of primary components potentially involved in ABA-mediated repression of grape bud dormancy release and in its removal

Investigators:  
Etti Or  
David Galbraith  
Ron Ophir  
Asaph Aharoni

Affiliated Institutions:  
ARO, The Volcani Center  
University of Arizona  
ARO, The Volcani Center  
Weizmann Institute

Start Date of Project:  October 1st, 2013
Date of Submission of Report: July 30, 2017

Signature  
Principal Investigator (PI)

Signature  
Institution’s Authorizing Official
Title: Characterization and manipulation of primary components potentially involved in ABA-mediated repression of grape bud dormancy release and in its removal

BARD grant IS-4639-13
2014 annual report (Final report)

Etti Or¹, David Galbraith², Ron Ophir¹, Asaph Aharoni³ (Volcani Center¹, U of A² and Weizmann Inst.³)

Project background

In warm winter regions, where the table grape industry preferentially is located, artificial induction of bud dormancy release is mandatory for coordinate, early production of economical grape yields. The single effective artificial stimulus available for commercial use in vineyards is hydrogen cyanamide (HC). The ability of HC to induce respiratory stress, which initiates a biochemical cascade that leads to effective dormancy release, is also, unfortunately, responsible for its toxicity, both to the vines and within the environment. These phytotoxic effects, which results in irregular ripening and significant yield losses, coupled with new regulations that have banned the use of HC in the deciduous trees industry from 2014, create an urgent need for the development of safe alternatives for artificial induction of bud dormancy release. Despite their high potential to serve as effective stimuli, searching for new chemical inducers of respiratory stress represents a short-term vision, in that conservation of mitochondrial function across eukaryotes invites toxic effects and future ban of its use. A much better strategy is to search for targets which are affected by the induction of respiratory stress and that regulate bud dormancy release, since future manipulation of these targets has a much better probability to be plant-specific and harmless to the environment.

Results of our genomic studies, previously funded by BARD, allowed us to propose a model for the cascade induced by artificial stimuli of dormancy release, which assigned central importance to the removal of ABA-mediated repression of meristem activity, and suggested a role for ethylene signaling in the regulation of this step. Further studies confirmed a transient induction of ethylene biosynthesis by various dormancy release stimuli, and demonstrated that ethylene is critical for the induction of grape bud dormancy release. We also found that exogenous application of ABA inhibits dormancy release, and that HC regulates expression of central components in ABA metabolism and levels of ABA and its metabolites. Being the last identified link in the chain of events that induce dormancy release, removal of ABA-mediated repression of dormancy release is the central theme of the current proposal. Based on our rationale, ABA targets that are repressed during bud dormancy, and core components which are potentially involved in removal of such repression and induce meristem activation, are the key regulators of growth resumption ability. Thus, their identification and characterization are prerequisites for controlled modulation of dormancy release, and their manipulation may become a useful tool for bud break control.
Towards achieving this goal, a combination of hypothesis-driven candidate gene analysis and a more broadly-based discovery approach was proposed.

In hypothesis-driven mode, we focused on selected core component of ABA metabolism, a bud-expressed ABA8ox, which is regulated by both HC and the natural dormancy cycle. Assuming this component may be involved in ABA-mediated repression removal, we characterized its response to other dormancy release stimuli, carried out its functional analysis, and successfully modulated bud dormancy by over expression of this gene in grapevines. A detailed description of our results appears in Chapter A1. We similarly characterized the potential involvement of selected components of ABA signaling, two ABRE genes (see Chapter A2).

In discovery mode, we used transcriptome profiling to potentially identify targets of ABA that may be related to the regulation of bud activation and components that are involved in the regulation of the natural dormancy. The analysis of the data, which is still ongoing and will not be addressed here in detail, will probably generate two future publications having a genomic viewpoint. Since the data revealed in this mode exposed potential core participants, we prefer to focus on those we already addressed in detail to obtain a broader understanding of core processes that are involved in bud dormancy release. Here, we describe the studies we carried out regarding the potential involvement of 1) GA metabolism (see Chapter B); 2) sugar metabolism (see Chapter C) and 3) and Jasmonic acid metabolism (see Chapter D) in the regulation of the dormancy cycle. Overall, the data revealed in the discovery mode provide a comprehensive “bud dormancy release atlas” that will be of great value in future research.

**RNA-seq analyses**

**Profiling the transcriptome following HC and ABA treatments**

Buds were collected from Control, ABA, HCABA and HC treatments as detailed in the grant proposal. 48 RNA samples were shipped from the Or lab to the Galbraith lab. Libraries were constructed and paired-end RNAseq was conducted. Data were transferred back to ARO and analyzed in the Ophir lab. Clipping, adapter removing, and low-quality filtering were performed specifically for each read by trimmomatic and FASTQC. That optimized a maximum data with maximum quality resulting in 97.44% read survivors. These reads were aligned to the *Vitis* genome reference using TopHat based on the GTF genome annotation file. An additional 20% were lost as a result of aligning to the genome reference. Read counts, normalization, and Bayesian correction were performed by the edgeR package. The high quality of biological replicates was revealed by clustering with multidimensional scaling (MDS) analysis. In those clusters, the control samples were clearly separated from the other samples at all time points. In addition, a significant difference of the ABA treatment from other treatments, and a partial difference between HC and ABAHC treatments were observed. Further statistical analysis of the factorial design was carried out, estimating the ABA effect, the HC effect, and the interaction between the two treatments on gene expression. A few global phenomena were observed. HC had the greatest effect on differential gene expression. ABA had a smaller effect, but with more up-regulated than down-regulated genes observed. The interaction between both had
the smaller effect and antagonistic effects was observed. All factors had the most pronounced effect after 24 hours.

**Gene selection**

Control, ABA, HC and ABA-HC treatments were applied, RNA was prepared from buds samples at 12, 24, 48 and 96 h, and RNA-seq was conducted.

To identify candidate genes that are significantly regulated by ABA and that have relevance to the inhibitory effect of ABA on HC- treated buds, we initially conducted two separate comparisons 1) ABA treatment vs. Control and 2) ABA-HC treatment vs. HC treatment. For each comparison, we conducted three filtering steps: (1) the raw data is filtered for genes that are significantly regulated at the same direction (Up or Down) for minimum of two-adjacent time points. We also specified that the other two time points should either be significantly regulated to the same direction or not significantly regulated, (2) Fold Chang (FC) of at least one of these two adjacent time points should be equal or higher than 1 (log2 value); (3) At the next step, we selected genes that were either regulated by HC in the opposite direction or not regulated by HC (in the pair HC/C), at each of the four time points.

We then combined the final lists from the two separate comparisons and removed duplicated genes. This resulted in a list of 132 genes - 123 genes that are up-regulated and 9 genes that are down-regulated by ABA (Table 1). Functions for these genes was assigned using MapMan software (Figure 1). Sixteen genes were assigned to stress (subgroup 20). A lesser number (7 to 9 genes) were assigned to cell wall, lipid metabolism, secondary metabolism, RNA processing/regulation and transport subgroups (subgroups 10, 11, 16, 27 and 34, respectively). Even fewer genes (1-3 genes) were assigned to photosynthesis, minor CHO metabolism, TCA, amino acid metabolism, metal handing, hormone metabolism, redox, nucleotide metabolism, protein, signaling, cell organization and development subgroups (subgroups 1, 3, 8, 13, 15, 17, 21, 23, 29, 30, 31 and 33, respectively). A group of 23 genes were assigned to miscellaneous (subgroup 26) and 48 genes had no assigned function (subgroup 35). Interestingly, seven out the nine down-regulated genes were found to be small heat shock proteins.

| Table 1 : Number of candidate genes that are significantly regulated by ABA |
|-----------------------------|-----------------------------|
|                     | Log2 > 1                   |
|                     | ABAHC vs HC                |
|                     | Up (122)                   |
|                     | Down (7)                   |
| Log2 > 1            | ABA vs C                   |
|                     | Up (5)                     |
|                     | 4                          |
|                     | 0                          |
|                     | Down (7)                   |
|                     | 0                          |
|                     | 5                          |
| Total               |                            |
|                     | 132 = 5 + 7 + 122 + 7 – 4 - 5 |
As an example of the relevance of the sorting process we bring *VvCPI*, a bud expressed ortholog of cysteine proteinase inhibitor which was up regulated by ABA treatment and down regulated by HC treatment. Interestingly, GA-induced cysteine proteinases (CPs), which degrade seed storage proteins and supply amino acids, are highly expressed during germination of rice (Watanabe *et al.*, 1991), barley (Mikkonen *et al.*, 1996) and wheat (Kiyosaki, *et al.*, 2007). Ethylene similarly activates the expression of a CP during the germination of chickpea (Cervantes *et al.*, 1994). On the other hand, ABA induced cysteine proteinase inhibitors (CPI) expression, in Arabidopsis (Seki, *et al.*, 2002), serving as negative regulator of seed germination due to inhibition of CP (Arai *et al.*, 2002). In agreement with such role, CPIs accumulated in maturating seeds (Kuroda, *et al.*, 1997) and its expression was down regulated in the meristems during the transition from the dormant to the non-dormant state of potato tubers (Campbell *et al.*, 2008). In light of the above, we speculate that ABA inhibiting effect on dormancy release may be transduced by induction of CPI, while stimuli of dormancy release inhibits its expression. It is interesting to note that, in parallel with the induction of *VvCPI*s expression, ABA may enhance accumulation of storage proteins as judged by induction of expression of six genes encoding for seed storage 2S albumin superfamily.

**Profiling the transcriptome throughout the natural dormancy cycle**

To compare the effects of artificial dormancy stimuli to that of the environment during the natural dormancy cycle, we performed RNA-seq analysis to follow the dynamics of the transcriptome.
RNA samples were taken from 10 time points (T) previously characterized as informative for the dormancy cycle (each represented by three biological replicates - R). These were used for RNA-Seq analysis (10Tx3R=30 samples) using the Illumina HiSeq-2000 platform.

**Preprocessing:** Raw data contained 744,286,704 read pairs. Estimation of read quality, clipping, and removal of low quality reads was done using the ‘Trimmomatic’ program. This program clips differentially the read edges based on a base call quality cut-off (Q) equal to 30. Due to the fact that low quality of reads is a result of low quality base calls mainly at the edge of the reads, specific clipping retain most of the reads whilst improving their quality. Reads having lengths less than or equal to 30 bp were removed (~2% of the reads). This left 732,773,308 read pairs for further analysis.

**Genomic annotation:** Gene expression was detected by counting either gene or transcript specific reads (counting was separately performed for each). Assignment of reads to a gene/transcript was done using TopHat and HTSeq. These programs align sequence reads to a reference genome using the “Bowtie” alignment program and a general transfer format (GTF) file. Only reads that aligned uniquely to a specific gene/transcript in the *Vitis vinifera* reference genome were counted. As a result, 85% of the reads were counted and associated with 29,971 genes.

**Clustering analysis**

To analyze the changes throughout the season, where no control is available, and since profiling over 10 time points was of interest, we developed an algorithm implemented in the ctsGE R-package. The package provided a way to explore the expression data based on hypotheses. A paper was published (Sharabi-Schwager M1, Or E1, Ophir R1, ctsGE - clustering subgroups of expression data, Bioinformatics, 2017). In short, the ctsGE R-package applies a sorting step which divides all of the data into small groups. The data is assigned to the different groups according to how the time points are related to the time-series median. Then clustering is performed separately on each group, in two steps: (1) scaling of each gene’s expression along experiment’s conditions is performed by a standard scaling function $[\text{time-median}(x)]/\text{mad}(x)$, where mad$(x)$ is the median of absolute deviation (MAD). Next, the area around the median of the gene-expression profile is calculated as the median bounded by higher and lower MAD units. After determining the limits around the gene-expression median, new index values are assigned at each time point: 0 if the standardized value is within the limits, 1 if it exceeds the upper limit and 1 if it exceeds the lower limit. Genes with the same index are grouped together, (2) each group of genes is clustered by k-means to create subgroups. The ctsGE package also provides an interactive tool to visualize and explore the gene-expression patterns and their subclusters. Filtering step before clustering has been suggested to be essential for cluster coherence by excluding the irrelevant genes in advance (Tritchler D. et al., Filtering genes for cluster and network analysis., BMC Bioinformatics, 2009) as well as to memory- and CPU-consuming of high-throughput data. ctsGE proposed a way of organizing and exploring expression data without eliminating valuable information. In the current project, we used these tools to group genes according to four hypothetical models of expression profiles relevant to the dormancy cycle: (1) Model A, describing genes that are
initially down regulated and later up regulated, mimicking the dormancy curve profile; (2) Model B, describing genes that are up regulated during dormancy induction or maintenance and down regulated during dormancy release, presenting an opposite profile to that of the dormancy curve profile; (3) Model C, describing genes that are down regulated during the dormancy cycle; (4) Model D, describing genes that are up regulated during the dormancy cycle. After the grouping step, we sub clustered genes within each of the A-D groups by the first time point they were regulated.

Here we focus on Model B, which we think reliably represents genes that are involved in repression, since their expression is reduced during initial repression removal. For example, we formerly described a similar expression profile for a bud-expressed NCED gene, which encodes for an enzyme that catalyzes ABA synthesis.

In Fig 2A, the expression profiles of the B-T3, B-T4, B-T5, B-T6 and B-T7 subclusters, containing 187, 448, 377, 283 and 80 genes, respectively) are presented. Functions were assigned to these 1375 genes by MapMan (Fig 2B), and it appears that T3 is characterized by a high percentage of stress, cell wall and signaling functions. The first two are down-regulated from T4 while signaling is down-regulated at T5. Cell organization and transport functions are highest at T4 and T5, while protein synthesis functions peak at T5 and T6. Interestingly, DNA synthesis/structure functions are higher at T7, at which point stress and signaling functions again increase. T3 and T7 might represent transition stages to the next status (T3 towards dormancy and T7 towards release), while T5-T6 may represents a stage of active attempts to maintain dormancy and/or prepare for dormancy release.

A smaller group (130 of these 1375 genes) is currently being studied in detail, this subset being selected based on function and a FC of 1.9 or above at the one of the two time points after expression induction. Of the genes in that list, some are related to calcium signaling, which we previously addressed (Pang et al., JExBot, 2007), and some to ABA metabolism or response which we address in Chapter A1 below. Interestingly, the group contains a Trehalose 6 phosphatase (TPP) gene which suggests a regulatory role for sugar signaling. We therefore started analyzing this aspect in further details as described in Chapter C. Three other interesting directions that are emerging are (1) the potential involvement of cell wall modifications, in light of dramatic change in the expression profile of 7 members of the xyloglucan endotransglycosylase 6 (XTR6) gene family, known to be involved in cell wall modification; (2) the potential involvement of Jasmonic acid signaling and metabolism in the regulation of the dormancy cycle, which is further analyzed as described in Chapter D; (3) the potential involvement of the meristem development regulatory gene Wuschel in the dormancy cycle. This aspect, which we have not addressed yet, appears as highly relevant, both since the meristem is the regulated entity within the bud and since GA:cytokinin appears to regulate bud burst, as discussed in Chapter B (GA). It is worth noting that based on the expression profiles, Wuschel interactors such as Clavata1, Clavata3 are also regulated during the natural dormancy cycle and in response to artificial stimuli of dormancy release.
Figure 2A

The figure shows a comparison of fold change across different conditions represented by T3 to T7. The x-axis represents the conditions, while the y-axis indicates the fold change.

Figure 2B

MapMan function distribution

The bar chart displays the distribution of functions across different conditions. Each condition (T3 to T7) is represented with a bar chart showing the percentage of occurrences for various functions such as Cell wall, Lipid metabolism, Amino acid metabolism, Secondary metabolism, Hormone metabolism, Stress, RNA processing/regulation, DNA synthesis/structure, Protein metabolism, Signaling, Cell organization/division, and Development.
Validation of RNA-seq results and use of RNA-seq data

Validation of numerous RNA-Seq profiles was done using RT-PCR, for both the natural dormancy cycle and the HC/ABA RNA-seq initiatives. The analyses indicated that the transcript profiles generated by RNA-Seq are very reliable, and allowed us to compile a long list of genes involved in ABA, GA, Jasmonate, and sugar metabolism, as well as a number of other genes which are regulated by dormancy status, as described in the following chapters. At this point, it is worth noting that, for many genes, we see differences between HC-induced dormancy release and the relevant corresponding stages in the natural dormancy cycle. We currently assume this may reflect differences between ecodormant buds sampled directly from the vineyard and HC-treated buds activated for growth under growth room conditions. Relevant RNA-seq data were used for hypothesis-driven studies described below.

Chapter A:
A1. Enhanced degradation of endogenous ABA enhance bud dormancy release (a paper is under revision for PCE)

We have formerly shown that treatment with exogenous ABA delays the dormancy release of grapevine buds (Zheng et al., JExBot, 2015). Observation of this inhibitory effect suggested that ABA might be an important component of the regulatory network that normally operates during the grapevine bud dormancy cycle. Hence, we proposed that both natural and artificial stimuli of bud dormancy release may exert their effect by decreasing endogenous ABA levels within the buds. We also proposed that such reduction in ABA level is achieved, at least partially, via upregulation of *VvA8H-CYP707A4*, the highest bud-expressed paralog of the gene family encoding ABA degrading enzymes, ABA 8′-hydroxylase (Fasoli et al., 2012; Zheng et al., 2015). In agreement with this proposition, we found that (1) several artificial stimuli of dormancy release (HC, HS, AZ) that trigger respiratory stress, induce *VvA8H-CYP707A4* expression; (2) *VvA8H-CYP707A4* transcript level is also modulated during the natural dormancy cycle, and markedly increased in amount at the transition from deep dormancy phase to dormancy release phase (Zheng et al. 2015). The objectives of current study were to test whether (1) *VvA8H-CYP707A4* expression is regulated by components activated downstream as a consequence of respiratory stress establishment, as proposed by the model, (2) *VvA8H-CYP707A4* functions as an ABA degrading enzyme, and (3) manipulation of *VvA8H-CYP707A4* expression will affect bud dormancy release.

Our experiments reveal that:

1. Imposition of hypoxia and application of ethylene, which are induced by artificial stimuli that lead to respiratory stress establishment, enhance expression of *VvA8H-CYP707A4* within grape buds (Fig. A-1). The results suggest that removal of ABA repression via upregulation of *VvA8H-CYP707A4* expression may occur downstream of the induction of respiratory stress. In agreement with the proposed
placement of anaerobic respiration in the cascade of events, treatment with exogenous ABA inhibited dormancy release of buds subjected to anaerobic conditions (Zheng et al., 2015).

2. Specific antibodies were produced and allowed to show that VvA8H-CYP707A4 protein accumulates during the natural transition to the dormancy release stage (Fig. A-2). The results support modulation of ABA degradation capacity during natural dormancy release, in agreement with formerly detected changes in ABA and its degradation products (Zheng et al., 2015).

3. Transgenic grapevine lines harboring CaMV 35S-driven overexpression of VvA8H-CYP707A4 were generated (Fig. A-3) which exhibit increased ABA catabolism in leaf and woody buds (Fig. A-4, 5) and significant enhancement of bud-break in controlled and natural environments, (= decreased endogenous ABA level enhance bud dormancy release) (Fig. A-6, 7). The results confirmed in vivo the function of the predicted activity and support the proposition that endogenous ABA inhibits dormancy release, and that its degradation removes such repression and enhances regrowth.

4. Interestingly, a phenotype of intensive outgrowth of summer laterals in basal nodes was observed in the VvA8H-CYP707A4 over-expression lines (Fig. A-8). When integrated with literature in the field, it appears that (1) regulation of outgrowth of grapevine summer lateral buds may be similar to that of the basal axillary buds of annuals which are inhibited by ABA; (2) regulation of activity of summer lateral and woody dormant buds may share similar regulatory steps, including ABA-directed repression of the meristem activation; (3) differences likely exist in regulation of the ABA level in these two types of buds, based on the extremely low level of the transcript of the bud-specific regulator of apical dominance, VvBRC1, in dormant woody buds, suggesting that VvBRC1 is not a player during the winter dormancy cycle (data not shown).

A2. Overexpression of the bud-expressed ABA response genes VvABF1 and VvABF2 attenuates bud dormancy release

We formerly recorded decreased expression of two bud-expressed ABA response genes, VvABF1 and VvABF2, in response to HC treatment (Zheng et al., 2015). To further test the potential effect of VvABF1 and VvABF2 on dormancy release, transgenic grapevine lines harboring CaMV 35S-driven over-expression of VvABF1 and VvABF2, were generated as described in Chapter A1 above. These lines, all confirmed to be transgenic (data not shown), exhibit significant attenuation of bud-break in controlled and natural environments (Fig. A-9, 10). The results support the assumption that ABA signaling has a role in inhibition of bud dormancy release.
Figure Legends - Chapter A

Figure 1: Effect of Ethylene and Hypoxia on levels of *VvA8H-CYP707A4* (*VvA8H4*) transcripts in grapevine dormant buds. Vines (*Vitis vinifera* cv. Early Sweet) from a vineyard at Gilgal, located in the Jordan Valley, were pruned to three-node spurs. The detached canes were cut into single-node cuttings, randomly mixed, and groups of 10 cuttings were prepared. Ethylene (100 ppm), KMNO₄ control (C+KM) and hypoxia (1% O₂) treatments were carried in sealed 2L jars (three jars per treatment as 3 biological replicates, 7 groups of 10 cuttings per jar, immersed in 150 ml water). Jars were placed in a growth chamber at 22 °C under a 14/10 h light/dark regime for 24 or 48 h. Nine groups of ten cuttings were placed in open vases with water under the same conditions and served as controls for the hypoxia treatment. Total RNA was extracted from buds sampled 24 and 48 h after treatment. Relative expression levels of *VvA8H-CYP707A4* were determined for Hypoxia (*A*) and Ethylene (*B*)-treated buds as well as their corresponding controls, using qRT-PCR normalized against *VvActin*. Values are averages of 3 biological replicates, each with 2 technical repeats. Bars represent SE.

Figure 2: Changes in the level of *VvA8H-CYP707A4* protein throughout the natural dormancy cycle of grapevine buds. Canes from the vineyard under study were sampled weekly through the dormancy cycle, and single-node cuttings were prepared as described for Fig. 1. Nine groups of 10 cuttings were forced weekly under the conditions described for Fig. 1. Bud break was monitored at 7, 11, 14, 18 and 21 d after forcing. Bud-break percentages at 21 d are presented to describe the seasonal changes in dormancy status of the bud population in the vineyard. Values are averages of nine groups, consisting of 10 buds each ± SE. Total proteins were extracted from buds sampled and frozen weekly, upon arrival from the vineyard. Western blot analysis of *VvA8H-CYP707A4* protein levels was carried out using a protein-specific, affinity-purified anti-*VvA8H-CYP707A4* polyclonal antibodies. Coomassie Brilliant Blue-stained (CBB) proteins was used as loading control. Band intensities were analyzed using ImageJ 1.48v software (Schneider et al. 2012). Underlined numbers below the western blot indicate the relative intensity of *VvA8H-CYP707A4* protein at each time-point compared to band intensity at 30-Oct-11.

Figure 3: Verification of over-expression of *VvA8H-CYP707A4* transgene in regenerated lines. Transgenic plants were generated by *Agrobacterium*-mediated stable transformation of cv. 101-14 Mgt embryonic calli using construct carrying the CaMV 35S-driven *VvA8H-CYP707A4* construct. Total RNA was extracted from young leaves of WT and regenerants, and cDNA was prepared and used as template for PCR and qRT-PCR. (A) Verification of transgene presence in 14 regenerants via PCR, using specific (*VvA8H-CYP707A4-F*) and vector (AttB2-R) primers, with an expected product size of 1556 bp. Plasmid DNA (of the construct used for transformation) was used as positive control. (B) Relative expression levels of *VvA8H-CYP707A4* were determined in leaves of the WT and 14 transgenic lines by qRT-PCR, and normalized against *VvActin* and *VvGAPDH*, as described in Fig. 1. Values are averages of 3 biological replicates, each with 2 technical repeats. Bars represent SE.

Figure 4: Effect of *VvA8H-CYP707A4* overexpression on levels of ABA and PA in young leaves. ABA (A) and PA (B) levels were determined separately in young leaves of WT and the fourteen transgenic lines described above. Separate analysis was carried for each line, each in three biological replicates. Samples were prepared and used for hormone extraction as detailed in Zheng et al., 2015. ³H-labeled ABA and PA were spiked as internal standards. Levels of ABA and PA were analyzed by liquid chromatography tandem mass spectrometry, as detailed in Zheng et al., 2015). The levels of the analyzed molecules were calculated from the peak area ratios of the endogenous molecule to the relevant internal standard. The presented values are the average of means of 3 biological replicates of each of 14 transgenic line and for 3 WT plants. Bars represent SE.

Figure 5: Bud of *VvA8H-CYP707A4* overexpression lines contain higher levels of *VvA8H-CYP707A4* transcript and protein and lower level of ABA. Five transgenic lines were selected for validation of the increased *VvA8H-CYP707A4* transcript (A) and protein (B) levels as well as decreased ABA (C) and increased PA levels (D) in dormant buds. Three randomly collected bud pools served a three biological replicates. For WT analyses, buds were pooled from 8 plants. For the transgenic lines, three buds pools were analyzed from each line. qRT-PCR were carried out as described in Fig. 3 with buds sampled in November 17, 2016. Values are averages of 3 biological replicates, each with 2 technical repeats. Western blot analyses were carried as described in Fig. 2. Levels of ABA and PA were determined as described in Fig. 4 in dormant buds sampled...
from the five selected lines at December 26, 2016. The presented values are the average of means of 3 biological replicates of each of 5 transgenic line and for 3 pools of WT plants. Bars represent SE.

**Figure 6: Effect of VvA8H-CYP707A4 overexpression on bud dormancy release of single node cuttings under controlled environment.** Detached canes (eight buds from positions 5-12) from WT and the 14 transgenic lines were used to prepare single-node cuttings. Cuttings from each cane were used as a biological repeat. Bud break was monitored as described in Fig. 2. Analyses were carried out on November 13, 2014 (A), December 8, 2014 (B), January 21, 2014 (C), November 18, 2015 (D), December 4, 2015 (E) and December 22, 2016 (F). Visual appearance, documented on December 31, 2014 for cuttings subjected to bud break analysis on December 8, 2014 (G). Values represent means ± SE of 14 transgenic plants and 4 WT biological replicates.

**Figure 7: Effect of VvA8H-CYP707A4 over-expression on bud dormancy release of vines.** Bud-break of cordon-trained transgenic and WT vines was monitored under natural conditions in the spring of 2016 (A) and 2017 (B). Visual appearance of 2 transgenic and 2 WT vines, documented on March 8, 2017 (C). Values represent means ± SE of 14 transgenic vines and 4 WT vines.

**Figure 8: Effect of VvA8H-CYP707A4 over-expression on lateral bud outgrowth.** (A) Visual presentation of 1-year old transgenic and WT vines. Total length (B) and number of nodes (C) of lateral shoots emerging from the 3rd to 18th node of the main shoot. Values represent means ± SE from 4 transgenic, and 4 WT vines.

**Figure 9: Effect of VvABF1 and VvABF2 overexpression on bud dormancy release of single node cuttings under controlled environment.** Bud break was monitored as described in Fig. 6. Analyses were carried out on November 13, 2014 (A), December 8, 2014 (B), November 18, 2015 (C) and November 17, 2016 (D). Values represent means ± SE of 17 (VvABF1) and 19 (VvABF2) transgenic plants and 4 WT biological replicates.

**Figure 10: Effect of VvABF1 and VvABF2 over-expression on bud dormancy release of vines.** Bud-break of cordon-trained transgenic and WT vines was monitored under natural conditions in the spring of 2016. Values represent means ± SE of 17 (VvABF1) and 19 (VvABF2) transgenic vines and 4 WT vines.
Chapter B: GA negates early meristem activation but required for growth (a paper is under revision for JExBot)

Our model, established by former BARD-supported research (Ophir et al., 2009), proposed that ABA represses bud-meristem activity, and perturbation of respiration induces an interplay between ethylene and abscisic acid (ABA) metabolism, leading to repression removal and Gibberellin (GA)-mediated growth resumption. While the first steps in the proposed cascade were addressed and supported by our previous work, the current study examines, for the first time, the involvement of GA in the naturally and artificially stimulated cascade that lead to dormancy release.

Our experiments revealed that:

1. During natural dormancy induction, expression of $VvGA3ox$ and $VvGA20ox$ genes (coding for GA biosynthetic enzymes) was decreased. During dormancy release, expression of these genes was enhanced, accompanied by a marked decrease of the bud-expressed $VvGA2ox$, which deactivates bioactive GA; the transcript profiles generally agreed with the changes in the level of endogenous active GA (Fig. B-1, 2). Overall, these results support the assumption that the cascade of events responsible for natural bud dormancy induction involves a decrease in GA biosynthesis capacity and a maintenance of GA degradation ability, which results in decreased levels of active GA. Maintenance of a low level of GA may thus be required during deep dormancy. They also support the assumption that increased GA synthesis capacity is required either during or after dormancy release. Such changes are achieved, at least partially, by regulation of transcription of GA biosynthesis and GA inactivation central regulators.

2. Based on the assumptions of the working model, and the aforementioned findings regarding changes in GA metabolism through the natural dormancy cycle, it was assumed that a supply of exogenous GA might bypass the need for regulated activation of GA biosynthesis during dormancy release, and serve as a direct stimulus of dormancy release. On the contrary, a concentration-dependent inhibitory effect of GA was documented, in both laboratory and field trials (Fig. B-3).

3. GA had no negative effect on bud burst when applied at 10 d after HC application (Fig. B-4A). This behavior suggests (1) that the inhibitory effect of exogenous GA is not a nonspecific, wide-range suppressive effect on bud primordial growth activity, (2) that the negative effect of GA on HC-treated buds is exerted mainly if GA is applied when the meristem is either still repressed or experiencing the initial steps of repression removal. However, if GA is applied once dormancy is released, it no longer has an inhibitory effect. The inhibiting effect of GA, when applied to pre-chilled buds or naturally ecodormant buds in parallel with forcing initiation, and its enhancing effect when applied seven and three days later, respectively (Fig. B-6B and 6C), further suggest that GA application during initial activation of the endo- or eco-dormant woody bud meristem, has a negative effect on meristem activation, while its application to an already activated meristem enhances regrowth.

4. The improved bud break of GA-treated buds in the presence of HC, as compared with buds treated only with GA (Fig. B-4A), suggests that HC may recruit the ability to manipulate the artificially increased levels of GA. In a wider view, preventing the rise of GA levels in the buds at the wrong time, possibly by temporarily affecting GA metabolism, may represent part of the ability of HC to advance
and increase bud break, independently from exposure to exogenous GA. In line with the above, a decrease in the transcript levels of several GA biosynthesis genes, and an increase in transcript levels of GA inactivation genes was indeed recorded 12-24 h after HC application (Fig.B-5D and 5E). The significant decrease in GA level at 48 h after HC application (Fig. B-4B) testifies that the effect of HC on transcription indeed leads to a temporary decrease in GA availability.

5. The effect of HC was actually multifaceted and was timing dependent, as a reversed effect was evident at 96 h (i.e. an increased transcript level of GA biosynthesis enzymes and decrease of GA inactivation enzymes, Fig B-5A-E). These opposite effects of HC, which depend on dormancy status, (assuming that at 96 h repression was removed) suggest that the enhancing effect of HC may involve optimized coordination of hormonal interactions during the cascade of events, which starts with meristem activation and continues with its re-growth. The validity of the timing-dependent reversed effects of HC on expression of GA biosynthesis and catabolism regulators is supported by similar effects recorded in response to other dormancy-release stimuli, such as hypoxia and HS (Fig. B-5F-O).

The nature of this inhibitory effect is not yet clear. Interestingly, GA represses numerous cytokinin (CK) responses. Among various antagonistic effects, it has been shown that GA constitutive signaling has a detrimental effect of on shoot apical meristem (SAM) function, which requires the presence of CK for establishment and maintenance of meristematic identity. A positive role for CK in regulation of paradormant bud outgrowth is also well established. In light of the above, we speculate that the GA inhibitory effect on grapevine bud break results from its negative effect on CK responses required for activation of the bud meristem. Our preliminary data show that isopentenyladenine (iP) levels are dramatically increased during natural and artificial dormancy release, supporting the assumption that CK has a role during grape woody bud meristem activation. Our preliminary data also show that GA application to dormant buds at the beginning of forcing process leads to a significant decrease of 33% in iP level at 96 h, compared with control (Etti Or, unpublished data). It was formerly suggested that mechanism of antagonistic effects of GA and CK has evolved to flexibly tune the balance between meristem activity, where high CK and low GA levels are required, and cell maturation, where high GA and low CK levels are needed. Similar logic may apply in the current case.
Figure Legends - Chapter B

Figure 1: Expression profile of genes coding for central components of GA metabolism throughout the dormancy cycle. Vines (*Vitis vinifera* cv. Early Sweet) from a vineyard at Gilgal, located in the Jordan Valley, were pruned to three-node spurs. The detached canes were sampled weekly throughout the dormancy cycle, cut into single-node cuttings, randomly mixed, and nine groups of 10 cuttings were prepared and placed in vases with water. The vases were placed in a growth chamber, at 22 °C under a 14/10 h light/dark regime. The bud-break percentages at 21 d are shown (as line) in each panel. Values are averages of nine groups of replications, consisting of 10 buds each ± SE. Total RNA were extracted from buds sampled and frozen weekly, upon arrival from the vineyard. Relative transcript levels were determined for *VvGA3ox1* (**A**), *VvGA3ox2* (**B**), *VvGA20ox3* (**C**), *VvGA20ox6* (**D**), *VvGA2ox3* (**E**) and *VvGA2ox4* (**F**), using qRT-PCR and normalized against *VvActin* and *VvGAPDH*. Values of qRT-PCR represent the mean ± SE of three biological repeats, each with two technical repeats.

Figure 2: Changes in the contents of GA1 throughout the dormancy cycle. Buds were sampled weekly as described in Fig. 1. Levels of GA1 were analyzed by liquid chromatography tandem mass spectrometry as detailed in Fig. A-4 (**C**). The levels of GA1 were calculated from the peak area ratios of the endogenous GA1 to its 2H-labeled internal standard. Values represent means ± SE of three biological repeats (10 buds per repeat).

Figure 3: The effect of GA3 application on bud break in single node cutting and whole vines. (A) Cuttings were sprayed with 1.25, 2.5, 5, 10, 20 and 40 ppm GA3 with 0.02% Triton X-100, or only 0.02% Triton X-100 solution (control), placed in vases and monitored for 116 d. For additional details see Fig. 1. (B) Cuttings were sprayed with 0.001, 0.01, 0.1 and 1 ppm GA3 and monitored for 28 d. For additional details see Fig. 1. (C) Vines were pruned to three-node spurs at January 14, 2014. GA (1 ppm GA3 with 0.02% Triton X-100 solution) and control (0.02% Triton X-100 solution) treatments were conducted at January 15. Four blocks of 3 vines were used and bud break was monitored separately for each vine in each block. The total number of buds was recorded and number of bursting buds was monitored in the field at February 24, March 4 and March 11. Values are averages of the twelve grapevines in the four blocks in each treatment ± SE.

Figure 4: The effect of GA3 on HC-triggered bud break. (A) For GA and GA-HC treatments cuttings were sprayed with 1, 5 and 10 ppm GA3 with 0.02% Triton X-100. In HC-GA treatment, cuttings were treated at the same time with HC (3% Dormex®). In Control treatment, cuttings were treated with 0.02% Triton X-100. All other details are as in Fig. 1. The bud break data recorded for 25 d after treatment are presented. Values are averages of nine groups of 10 cuttings in each treatment ± SE. (B) GA1 levels were determined in control and HC-treated buds sampled at 48 and 96 h after treatment as described in Fig. 2. Values represent means ± SE of three biological repeats (10 buds per repeat).

Figure 5: The effect of artificial stimuli of dormancy release on expression profile of central components of GA metabolism. Total RNA was extracted from control (0.02% Triton X-100), HC (3% Dormex® with 0.02% Triton X-100) and HS (50 °C water for 1 h)-treated buds sampled at 12, 24, 48 and 96 h after treatment, and from hypoxia (1% O2)-treated buds and corresponding control buds at 48 and 96 h after treatment. Relative expression levels of *VvGA2ox3*, *VvGA3ox1*, *VvGA3ox2*, *VvGA20ox3* and *VvGA20ox6* were determined by qRT-PCR in HC (**A-E**), HS (**F-J**) and hypoxia (**K-O**) treated buds, and normalized against *VvActin* and *VvGAPDH*. Values represent the mean ± SE of three biological repeats, each with two technical repeats.

Figure 6: The effect of the timing of GA3 application on bud break. (A) GA treatment was applied at 0, 2, 4, 6, 8 and 10 d after HC treatment. For additional details see Fig. 4A and Fig. 1. (B) Canes were collected at November 22, 2015, pre-chilled at 4 °C for 1000 h and used to prepare groups of cuttings as described in Fig. 1. Nine groups of 10 cuttings were immediately treated with GA (1 ppm GA3 with 0.02% Triton X-100). The rest of the cuttings were treated with 0.02% Triton X-100. Nine groups of 10 cuttings were used as control and nine groups of 10 cuttings were treated with GA 7 days later as described above. For additional details see Fig. 1. (C) Canes were collected in the vineyard after endodormancy release (February 18, 2016). The experiment was designed and operated as described in B. The delayed GA treatment was carried out after 3 days. Values are averages of the nine groups in each treatment ± SE.
Chapter C: Induction of changes in sugar metabolism may participate in dormancy induction and release (MA thesis was published. Additional experiments are required for future publication)

According to the suggested cascade, damage to the mitochondrial oxidative phosphorylation process leads to respiratory stress, involving an increase in glycolytic activity and a temporary transition to anaerobic respiration. Such stress then leads to ethylene production and signaling, which in turn leads to removal of ABA repression, bud dormancy release and, eventually, resumption of bud growth. The existence of the respiratory and oxidative stress and the hormonal changes described in the model were uncovered by a series of experiments, but the link between the stress and the hormonal modules remains unclear.

To test the assumption that interference with respiration induced by artificial dormancy release stimuli leads to a change in sugar metabolism, which may later serve as a signal that activate changes in the hormonal balance, we analyzed (1) the changes in expression of genes involved in sugar metabolism across the natural dormancy cycle and in response to artificial dormancy release stimuli; (2) the level of selected metabolites relevant to sugar metabolism across the natural dormancy cycle and in response to HC.

Our experiments revealed that:

1. Expression levels of Hexokinase2 (HEX2), phosphofructokinase (PFK) and pyruvate kinase (PK) - genes involved in glycolytic cycle - are upregulated during natural dormancy induction, with a maximal level of upregulation achieved at deepest dormancy (Fig. C-1). Similar expression profiles were recorded for two additional groups of genes:
   - Alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH) and pyruvate decarboxylase (PDC) - involved in anaerobic respiration (Fig. C-1).
   - Invertases (INV) and a sucrose synthase (SUSY) - involved in sucrose degradation (Fig. C-2)

Notably, a sharp decline in the level of transcripts of all these genes was recorded shortly after the arrival of the buds at their maximal depth of dormancy. Normalized reads are presented here which were validated by RT-PCR for selected genes and suggest that RNA-seq results are of high quality.

It was formerly assumed that anaerobic respiration will operate during dormancy release, and will act as an inducer of the cascade that leads to bud break. However, the peak of PDC, ADH and LDH transcripts at maximal dormancy depth suggest that respiratory stress may be induced in advance of dormancy release, leading to an anaerobic respiration which serve as initial, essential, and inherent trigger for this process. To allow it, enhanced glycolytic activity is required to supply pyruvate and reducing power. An increased need for glucose for anaerobic respiration requires sucrose degradation.

Several dormancy release stimuli (HC, sodium azide, and hypoxia) upregulated LDH, ADH and PDC expression 12 hours after treatment. This was accompanied by an increase in gene expression of
HEX2, PFK and PK, and followed by increased expression of INV and SUSY. These changes (data not presented) similarly suggest increased glycolysis in response to respiratory stress that is induced by the three dormancy release agents, and activates anaerobic respiration.

The similar changes described during the natural dormancy cycle and in response to dormancy release agents suggest causal connections between respiratory stress, anaerobic respiration, changes in sugars metabolism and bud dormancy release. Based on expression of the relevant genes, starch decomposition does not appear to occur and be a part of the mechanism that induces dormancy release (Fig. C-4).

2. Levels of various sugars, acids, amino acids and starch were monitored across the natural dormancy cycle (Fig. C-2, 3). Sucrose levels were low, but started to increase towards dormancy release in parallel with increased expression of sucrose-P-synthase (SPS) which catalyzes sucrose synthesis (Fig. C-1). Similar profiles were recorded for glucose and fructose (Fig. C-1). Milder increases were recorded for phosphorylated glucose and fructose, whose levels are three magnitudes lower (Fig. C-3). Starch levels are low at the beginning of the natural dormancy cycle and mainly increase towards dormancy release (Fig. C-4). Starch levels were not markedly affected by HC (data not shown). Interestingly, the level of trehalose, a degradation product of the activity of trehalose 6-phosphatase (T6P), was increased to its maximum at the stage of deepest dormancy (Fig. C-3).

3. Downregulation of trehalose phosphate synthase1 (TPSI) expression was observed during entry to dormancy and its upregulation was recorded during dormancy release. Trehalose phosphate phosphatase C (TPPC) expression presented the opposite profile, in agreement with increased level of trehalose (Fig. C-5). Together, these changes may hint at a decrease in the levels of trehalose-6-phosphate (T6P) during dormancy induction and its increase after dormancy release. The decrease in TPS1 expression correlated with increased expression of sucrose degradation genes, while the increase of TPS1 during dormancy release was correlated with an increased level of sucrose.

It was shown before that increases in sucrose level lead to an increase in T6P, which inhibits the activity of the kinase SnRK1, a repressor of growth related gene expression. Integration of the data presented here and found in the literature suggests a speculative cascade (Fig. C-6) in which increased dormancy depth leads to a respiratory stress, which increases anaerobic respiration that enhances sucrose degradation which leads to decrease in the amount of T6P. The decreased level of this signal molecule then activates SnRK1, which inhibits meristem growth. After maximal dormancy was reached, the decrease in anaerobic respiration and the increase in sucrose and T6P levels renews inhibition of SnRK1 activity which allows activation of meristem growth through increased expression of growth activators. The potential involvement of SnRK1 in dormancy activation is supported by a parallel rise in ABA, a known activator of SnRK1, during dormancy induction.
Figure Legends - Chapter C

Figure 1: Expression profile of genes coding for central components of glycolysis and anaerobic respiration throughout the dormancy cycle. Buds from detached canes were sampled weekly throughout the dormancy cycle as described in Fig. B-1. Total RNA was extracted from buds frozen weekly, upon arrival from the vineyard. Relative transcript levels were determined for VvHex (A), VvPFK (B), VvPK (C), VvLactate DH (D), VvAlcoholDH (E) and VvPDC4 (F), using RNA-seq. Profiles were validated by qRT-PCR for selected genes (data not shown). Normalized read values represent the mean ± SD of three biological repeats.

Figure 2: Profiling levels of sucrose, fructose, glucose and transcripts of components in sucrose metabolism throughout the dormancy cycle. Levels of sugars were determined by GCMS as described by Roessner et al. (2003), with ribitol as an internal standard. Retention times and mass spectra were used to identify the molecules, and quantities was determined against that of ribitol. All other details are as in Fig. 1. Relative transcript levels were determined for SUSY (A), VvINV (B), VvSPS (C), Sucrose (D), Fructose (E) and Glucose (F).

Figure 3: Profiling levels of various sugars throughout the dormancy cycle. All other details are as in Fig. 1.

Figure 4: Profiling levels of starch and transcripts of components in starch metabolism throughout the dormancy cycle. Starch levels were determined using the Starch (HK) Assay Kit (Sigma SA-20). All other details are as in Fig. 1. Relative levels were determined for VvBetaAmy (A), VvADP-phosphrylase (B), VvSoluble starch synthase (C) and Starch (D).

Figure 5: Expression profiles of genes coding for central components of trehalose 6-phosphate metabolism. All other details are as in Fig. 1. Relative levels were determined for VvTSP1 (A) and VvTPPc (B).

Figure 6: Hypothetic connections between sucrose, T6P and ABA metabolism and levels and their potential involvement in regulation of the bud meristem activity.
Chapter D: Potential involvement of Jasmonic acid (JA) in regulation of dormancy status (validations will be carried in the coming season to allow publication)

JA biosynthesis starts within the chloroplast, where OPDA synthesis is regulated by 13-LOX, 13-AOS, and AOC. cis-OPDA can directly serve as a nuclear signal, or can be used as a precursor for JA synthesis in the peroxisome (sequentially regulated by OPR, OPCL, and ACX). The changes observed in the levels of the chloroplastic precursors octa and hexadecanoic acids and the existence of LOX, AOS and AOC in the selected group from the B model genes in T3 to T5 suggested the JA may have a role in dormancy during dormancy induction and/or maintenance. So far we (1) followed profiles of the bud expressed members of the above mentioned genes in the JA metabolic pathway and JA signaling, and present here a representative selection, (2) quantified endogenous JA level in natural and HC treated buds, (3) tested the effect of exogenous application of JA and methyl-JA on dormancy release at selected time points across the dormancy cycle. This led to further investigation of regulation of this pathway and its products.

Currently, it appears that: (1) during the natural cycle, the levels of genes coding for most enzymes are up-regulated at dormancy and down-regulated during release (Fig. D-1A, C, E, G, I and M); (2) the level of ACX, the regulator of the final step that converts OPC-8-CoA to JA, is regulated inversely to the above during the natural dormancy cycle (Fig. D-1K); (3) the changes in levels of JA and JA-Ile are enhanced during the early stage of dormancy induction, then decreasing until maximum dormancy is reached, and increasing again once dormancy release is initiated (Fig. D-3); (4) treatment with HC led to increased expression of all the above mentioned genes except LOX3 and JAR2, which are down regulated (Fig. D-1B, D, F, H, J, L, N and P); (5) HC treatment also led to a very significant increase in JAR1 levels (which transforms JA to Ile-JA before it can serve as a nuclear signal); the latter observation is in agreement with the observed decrease in JA following HC treatment that was accompanied by an increase in Ja-Ile levels (Fig. D-4), which also is consistent with the observation of increased JA-Ile during natural dormancy release; (6) expression levels of the signaling regulator GAZ and of JAI homologs were up-regulated during dormancy induction, and were down-regulated during natural dormancy release and 12-24 h after HC application (Fig. D-2), and (7) both Me-JA and JA inhibit bud break in a concentration-dependent manner, and that bud sensitivity is a function of its dormancy status (Fig. D-5). Interestingly, it appears that whereas ABA treatment had no effect on expression of the chloroplast-related JA biosynthesis genes, it appears to limit the up-regulation of the peroxisome-related JA genes and JAR1.

We have not yet pinpointed the location of the JA effect within our working model. We plan to further test key results in the coming seasons, prior to proposing the mechanism by which JA regulates bud behavior.
Figure Legends - Chapter D

Figure 1: Expression profile of bud expressed genes coding for enzymes of JA biosynthesis pathway across the natural dormancy cycle and in response to HC. Relative transcript levels were determined for *VvLOX3* (A and B), *VvAOS* (C and D), *VvAOC4* (E and F), *VvOPR4* (G and H), *VvOPCL2* (I and J), *VvACX* (K and L), *VvJAR1* (M and N) and *VvJAR2* (O and P), using RNA-seq. Normalized read values represent the mean ± SD of three biological repeats. For additional experimental details see Figs. B-1 and B-5.

Figure 2: Expression profiles of genes coding for central components of the JA signaling across the natural dormancy cycle and in response to HC. Relative transcript levels were determined for *VvJAZ2* (A and B) and *VvJAI1* (C and D), using RNA-seq. Normalized read values represent the mean ± SD of three biological repeats. For additional experimental details see Figs. B-1 and B-5.

Figure 3: Changes in the contents of JA and JA-Ile across the dormancy cycle. Levels of JA and JA-Ile were analyzed by liquid chromatography tandem mass spectrometry as described by Zheng et al., 2015, JExBot. Additional details are provided in Fig. A-4. The levels of JA and JA-Ile were calculated from the peak area ratios of the endogenous JA and JA-Ile to its 2H-labeled internal standard. Values represent means ± SE of three biological repeats (10 buds per repeat).

Figure 4: Changes in the contents of JA and JA-Ile in response to HC application. (A) Changes in the levels of JA in response to HC application. (B) Changes in the levels of JA-Ile in response to HC application. See details in Fig. 3.

Figure 5: The effect of Methyl Jasmonate and JA application on bud break in single node cuttings. (A) Cuttings were sprayed with 1, 2.5, 5, 10, 25, 125 and 250 µM MeJA with 0.02% Triton X-100, or only 0.02% Triton X-100 solution (control), placed in vases and monitored for 21 or 24 d. For additional details see Fig. A-1 and B-3. (B) Cuttings were sprayed with 1, 2.5, 5, 10, 25, 125 and 250 µM FiJA with 0.02% Triton X-100, or only 0.02% Triton X-100 solution (control), placed in vases and monitored for 21 or 24 d. For additional details see Fig. A-1 and B-3. Values are averages of the nine groups of replications, consisting of 10 buds each ± SE.
Figure 5