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Expression of the genes *(CBF2, CBF4, NCED1, MYB14, PLD)* during acclimation and deacclimation in dormant buds of Chambourcin and 3309 C grapevine accessions

Dilek KARATAŞ*, **Hüseyin KARATAŞ**
Department of Horticulture, Agriculture Faculty, Dicle University, Diyarbakır, Turkey

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Abstract: Deacclimatization results in a loss of bud cold-hardiness whereas cold acclimation results in an increase in it. The regulation of the buds' cold acclimation and deacclimation in woody plants are poorly understood. This study indicated that differential gene expression during acclimation and transition of the buds from acclimation to deacclimation in the light of dormancy and bud water content. One-year-old vine latent bus of cv. Chambourcin vines and 3309 C (*Vitis riparia* x *Vitis rupestris*) were used for the analyses. Five genes (*CBF2, CBF4, NCED1, MYB14*, and *PLD*) were selected for quantitative real-time PCR.
Quantitative real-time PCR was applied to measure gene expression of the dormancy related genes and *GAPDH* gene was used as a reference gene. *CBF2, NCED1*, *MYB14,* and *PLD* were strongly upregulated by both with the two-temperature treatment (acclimation (5 °C) and deacclimation (15 °C)) and two different time applications (one and two week) while the expression of *CBF4* was low after the applications. Among these four upregulated genes, *NCED1* and *PLD* exhibited the highest level of expression (121.02 and 86.40, respectively) at cold hardy 3309 C during deacclimation (T2, 15 °C). However, for the 5 °C treatment, Chambourcin showed the highest level (32.61) of *MYB14* gene expression during T2 application, while 3309 C buds had the highest *NCED1* gene (94.83) expression level again during T2 application. According to the results, it is clearly highlighted that *PLD* and *NCED1* gene expression level was highest at cold hardy 3309 C during dormancy and release of dormancy.

Key words: Dormancy (D50BB), gene expression, qRT-PCR, transcription factors, *Vitis*

1. Introduction

Grapevine is one of the most important fruit crops in the world. In autumn, grapevines enter the dormancy process. This process continues until spring when growth and development activity starts again. During the cold tolerance process of grapevines, dormancy is associated with three physiological stages; paradormancy, endodormancy, and ecodormancy. During ecodormancy, the grapevines begin to lose their cold tolerance (deacclimate) as they tread just to warmer temperature conditions (Lang et al., 1987).

Cold acclimatization is a complex process that includes many biochemical and physiological changes and consequently affects the expression of regulatory and functional genes (Fennell 2014; Wisniewski et al., 2018). Among regulatory genes, several transcription factors play an important role in plant stress responses. These factors act as coordinators of stress signals and regulate the expression of functional genes (Wang et al., 2016).

Abscisic acid (ABA) is recognized as an essential phytohormone in dormancy regulation, but its regulatory mechanism in bud dormancy is not well understood (Pan et al., 2021).

ABA plays an important role in grapevine dormancy from the initiation of cold acclimation in late summer through the release from dormancy in early spring. Gene expression data indicate that ABA metabolism might be always inversely correlated with grapevine vegetative growth, which means that ABA catabolism is activated when the grapevine actively grows, and ABA synthesis is activated when the grapevine is dormant (Wang, 2019; Rubio and Pérez, 2019a).

C-repeat-binding factor (*CBF*) *dehydration responsive element binding* (*DREB*) protein (*CBF/DREB*) transcription factors are well known to play a role in the development of cold resistance (Xiao et al., 2006; Xiao et al., 2008; Thomashow, 2010; Theocharis et al., 2012). The transcription factors encoded by CBF⁄DREB family members described for *V. vinifera* and *V. riparia* were found to regulate genes that respond to low temperature, drought stress, and exogenous ABA application (Xiao et al., 2006). These transcription factor genes (*VvCBF1*, *VvCBF2, VvCBF3, VvCBF4*) showed increased expression exposure to freezing and drought stresses (Xiao et al., 2006; Xiao et al.,

* Correspondence: dilek.degirmenci@dicle.edu.tr

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2008). Although known as central in senescence and dormancy, the role of ABA in bud dormancy is not fully understood. The *VvCBF* transcription factors can control the expression of genes related to all aspects of the cold-acclimatization process in grapevine dormant buds (Tillet et al., 2012; Londo and Garris 2014). Global transcriptome analyses have revealed that ABA is one of the key players in response to cold in grapevine (Londo et al. 2018; Rubio et al. 2019b).

NCED gene *(putative 9-cis-epoxycarotenoid dioxygenase)* plays an important role in ABA biosynthesis in plants, minimizing damage to plants as a defense mechanism against environmental stress such as drought and salt stress (Wang et al. 2019). It has been determined that the *NCED1* gene is expressed in 3 homologous genes during dormancy (Zheng et al. 2015).

The lagest transcription factors (TFs) family in grapevines, *Vitis vinifera R2R3 MYB14* proteins were shown to be involved in response of hormone and environmental factors (Höll et al. 2013; Zheng et al. 2015; Min et al. 2017). Min et al. (2017), to better understand the molecular mechanisms behind this, the transcriptomes of grapevine (*Vitis vinifera*) latent buds and prompt buds were analyzed using RNA-sequencing (RNA-seq) technology and compared. The highest number of *MYB* (TFs) were identified to be candidates regulating grapevine bud dormancy.

Recent RNA-sequencing studies related to the dormancy in vines demonstrated many other TFs, *WRKY* (Jiang et al., 2017), *DAM* (*Dormancy Associated Mads-Box)* (Díaz-Riquelme et al., 2012; Shim et al., 2014; Liu and Sherif, 2019), *SVP (Short Vegetative Phase)* (Min et al., 2017; Falavigna et al., 2019) genes involved in the control of bud dormancy in grapevines.

Phospholipase D (*PLD*) belongs to a large gene family widely found in plants; it can hydrolyze phospholipids to produce phosphatidic acid (PA) and free polar head groups. The PLD family plays an important role in response to various stresses, such as programmed cell death, freezing tolerance, drought, and salt tolerance (Kovaleski and Londo,2019). With the NGS (New Generation Sequencing) illumina gene sequencing analysis by Kovaleski and Londo (2019), the *PLD* (*Phospholipase D*) gene as a new gene family that is upregulated in the process of exiting from ecodormancy and dormancy was highlighted in the study.

Increased bud cold-hardiness indicates cold acclimatization, while decreased bud cold-hardiness indicates deacclimation. Since genotypic variation is crucial for climate adaptation, it is unclear what processes underlie the buds' acclimation and switch from acclimation to deacclimation (Rubio and Pérez 2019b; Kovalesky and Londo 2019). In this study, our aim is to examine the expression level of *CBF2*, *CBF4*, *NCED1*, *MYB14* and PLD genes in Chambourcin and 3309 C (*Vitis vinifera* × *Vitis riparia*) dormant vine buds during cold acclimation and deacclimation processes under controlled growth chamber (GC) conditions.

2. Material and methods

2.1. Research vineyard

The OSU (The Ohio State University) Research Vineyard at OARDC (Ohio Agricultural Research and Development Center) in Wooster, Ohio, is the vineyard where the study was carried out (lat. 40°44'16" N; long. 81°54'12" W). Elevation: 355 m above sea level USDA. At the vineyard the space between row and vine was as 274.32 cm × 182.88 cm. The grapevine's trellis system had a bilateral low cordon (1 m in height) and vertical shoot position (VSP). Prunning was done to 30 buds on each vine as spur pruning (two to three buds per spur).

2.2. Experimantal design

One-year-old grapevine cane with latent buds of 3309 C (*Vitis riparia* × *Vitis rupestris*) (tolerant to cold stress) rootstock and Chambourcin (moderately tolerant to cold stress) grapevine canes harvested and used for treatment. Canes were collected from 3 vines (reps) each with 12 buds (node 3-12) from OARDC Research Vineyard/ OSU on April 1st 2019. The temperature was –2/–5 °C (https:timeanddate.com). Canes were bundled together, and stored at air temperature (without any cooler), and brought in the laboratory, and stored at room temperature. Pruned to 1-node cuttings and trays were placed in a GC (Conviron, Pembina, ND) with the following settings: 5 °C (acclimation) and 15 °C (deacclimation) for 12-h photoperiod with 300 μmol·m-2·s-1, and 70% relative humidity (Zhang and Dami, 2012). Twelve buds were used for each replication and a total of 36 single cuttings with 3 replications used for each experiment (water content, bud dormancy, RNA extraction analyses). Canes with visible periderm formation were excised into one-node cuttings ~5 cm long, then inserted into 2.5 cm × 2.5 cm foam medium (Smithers-Oasis, Kent, OH) and placed in 55 cm × 25 cm × 7 cm plastic trays 58 (T.O. Plastics, Clearwater, MN) filled with deionized water (Zhang and Dami, 2012).

2.3. Dormancy (D50BB)

Trays from GC 5 °C and 15 °C, moved to 23 °C GC and 80% relative humidity after one week (T1) and two week (T2) exposure to observe bud burst and D50BB at the date of 08.04.2019 and 15.04.2019, respectively. Budburst was recorded as three times per week for 30 days (Eichhorn and Lorenz, 1977). Dormancy results were estimated to be 50% bud burst as days (D50BB), with higher D50BB values indicating more dormant buds (Wake and Fennel, 2000).

2.4. Water content

Bud samples were collected separately from the growth chamber for acclimatization (5 °C) and deacclimation (15

°C) testing. The canes were put in plastic bags with damp paper towels to prevent drying out. The buds of each shoot were excised and weighed immediately after collection. The bud samples were placed in an oven at 70 °C for one week, and then the dry weights of the bud samples were measured. Bud water content was analysed using the protocol described by Zhang et al. (2011).

2.5. qRT-PCR analysis

For qRT-PCR analysis buds samples were collected from GC 5 °C and 15 °C at one-week and second-week applications. Bud samples were excised and then placed in 2 µL tubes outside with liquid nitrogen. Then immediately buds were placed at –80 °C until further use. RNA was extracted using the method previously described by Gambino et al., (2008), for extraction from grape buds with some modifications. DNA was removed by free DNase (Sigma) application. Primers were designed based on the information registered with NCBI. First strand cDNAs were produced from 1 µL (100 ng/µL) of total RNA using the abm® qRT-PCR cDNA Synthesis kit according to the manufacturer's instructions. Five genes (*CBF2*, *CBF4*, *NCED*1, *MYB14*, *PLD*) were selected for using quantitative real-time PCR. To identify *Vitis vinifera* genes, they were identified from the *V. vinifera* genome based on their annotation against the NCBI database. Primers were designed for each of the genes using the web page Integrated DNA Technologies (IDT) and NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/ primer-blast) (Table 1 and 2). Primer concentration was optimized using combinations of 100 mM forward and reverses primers. Using PerfeCTa® SYBR® Green qRT-PCR master mix (Quanta bio), 10 µL reactions were set up in duplicate in 96-well plates following the manufacturer's instructions. One µL of synthesized cDNA diluted 1:10 was used as a template. The qRT-PCR reaction was performed in a total volume of 10 µL, containing 1 µL of diluted cDNA, 0.3 µL of reverse and forward primers, 3.4 µL of ddH_2O, 5 µL SYBR (2x). According to the standard protocol of the ABI 7300 real time PCR system, the amplification program was performed as follows: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C for and 30 s at 60 °C. To verify the formation of single peaks and to exclude the possibility of primer dimer and nonspecific product formation, a melt curve (15 s at 95 °C, 60 s at 60 °C, and 15 s at 95 °C) was generated by the end of each PCR reaction. All reactions were performed in three biological replicates, each with three technical replicates, including the nontemplate control reactions. Each reaction had *GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)* reference gene. In addition, the threshold cycles (Ct) of the triplicate reactions for each tested gene were averaged, and then the values were normalized to that of the control *GAPDH* gene. Data were analyzed in CFX ManagerTM software (Bio-Rad, Hercules, CA) using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Data analysis

Gene expresions data were subjected to a three-way ANOVA, time, temperature and genotype were fixed factors. Means separated by Tukey HSD multiple comparison test after ANOVA. The analysis of variance (ANOVA) was performed using the software SPSS (v26) and a graph was constructed using Excel (2013) software.

3. Results

3.1. Bud dormancy analysis

Bud break (% BB) analyses of grape buds during acclimation and deacclimation are presented in Figure 1 and 2. Bud breakage started approximately 15 days after single bud cuttings were transferred to GC. Bud breakage for D50BB (days to 50% bud burst) was 13 days (5 °C, one week) and 14 days (5 °C, two week) during acclimation and 11 day (15 °C, one week) and 10 day (15 °C, two weeks) for 3309C. D50BB (days to 50% bud burst) were as 11 day (5 °C, one week) and 14 day (5 °C, two week) during acclimation and 11 day (15 °C, one week) and 7 day (15 °C, two week) for Chambourcin (Figure 3).

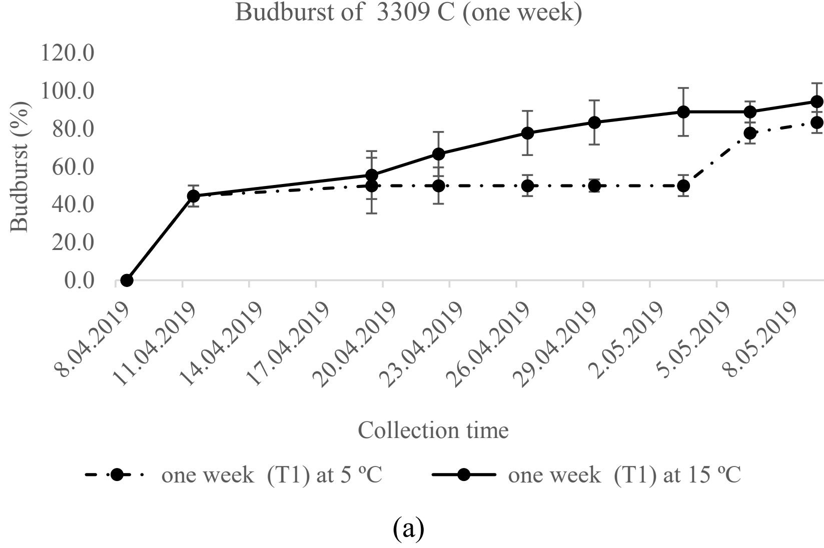
Table 1. List of putative *Vitis vinifera* genes used for qRT-PCR analyses.

Gene	Protein	NCBI*	GI	Organism	Reference
VvCBF2	*C-repeat binding factor 2*	KR2332931	939193631	*Vitis vinifera*	Xiao et al. 2006
VvCBF4	*C-repeat binding factor 4*	JN566061	343459402	*Vitis vinifera*	Xiao et al. 2008
PLD	*Phospholipase D alpha*	KT779428.1	951311831	*Vitis vinifera*	This study
NCED1	*9-cis-epoxycarotenoid dioxygenase 1*	AY337613.1	38569165	*Vitis vinifera*	Zheng et al. 2015
MYB14	*R2R3 Myb-related transcription factor*	EU181424.1	158323775	*Vitis vinifera*	This study
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	EF192466.1	122893269	*Vitis vinifera*	This study

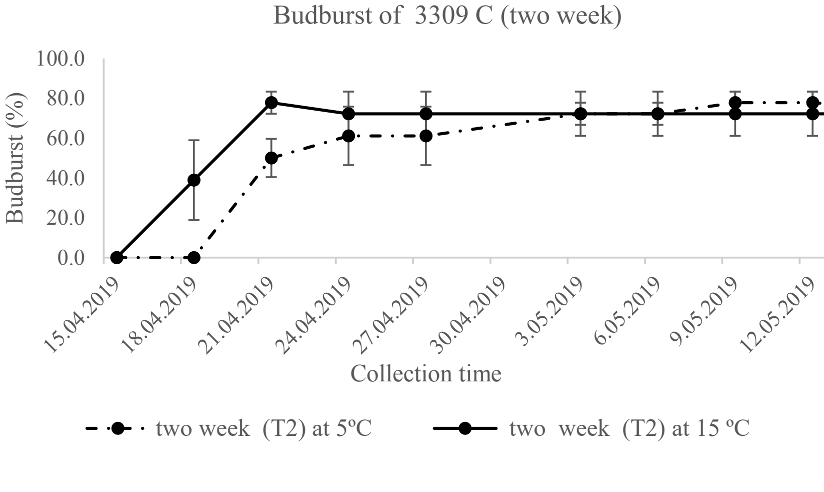
*NCBI: https://www.ncbi.nlm.nih.gov.

Table 2. Genes and primer pairs used for qRT-PCR.

Gene	Forward	Reverse	Annealing temp. (°C)
VvCBF2	GGGTGAGCACAAACATCTATGA	GAAGGTGAAGAAAGCCAAGAAAG	55
VvCBF4	CGACGCCAAGGACATTCA	CTCTCTTCCCTCCTCTCATCTT	55
PLD	GAAAGCAAGGTGGGAAGGATA	CTGGTCATCAGGGAACATAACA	55
NCED1	ACCACACTCCCAAAAGAGAAGG	TTAGGAAGAGAGGGTGCTGGGT	55
MYB14	GGGAGGACAGACAATGAGATAAA	GCTGAGGAGATATCGCTAAAGG	55
VvGAPDH	TCAAGGTCAAGGACTCTAACACC	CCAACAACGAACATAGGAGCA	55



(a)



(b)

Figure 1. Budburst analysis of 3309 C grape buds during all collection times (a: one week (T1); b: two week (T2)).

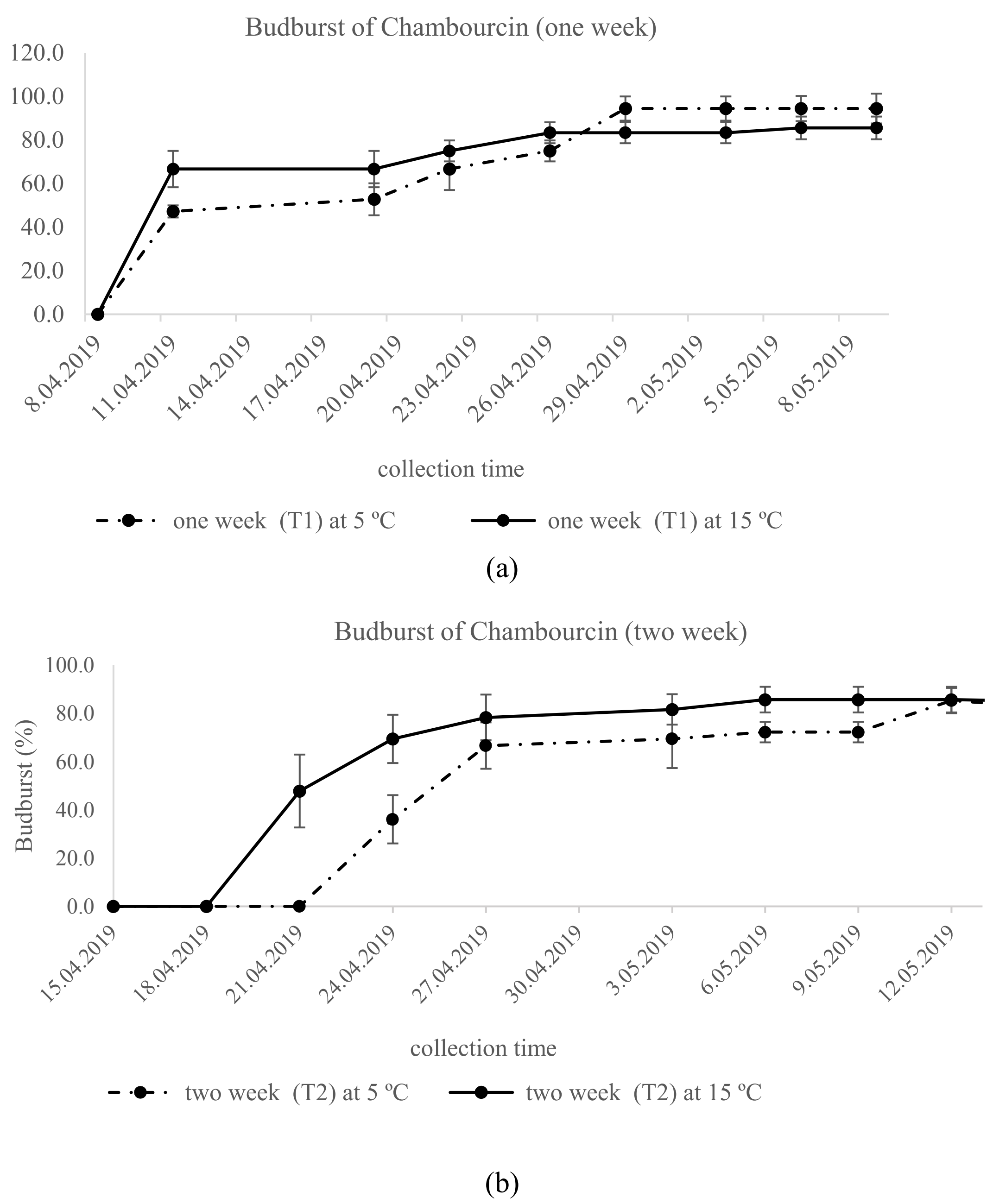


Figure 2. Budburst analysis of Chambourcin grape buds during all collection times (a: one week; b: two week)

3.2. Bud water content analysis

The water content analyzes of grape buds during acclimation and deaclimation results are presented in Figure 4. Changes in water content in vine buds during dormancy are associated with an increase in cold tolerance. With the induction of dormancy and during the acclimation period, the water content in the buds decreases. The water content of Chambourcin decreased with time at 5 °C and did not change statistically in 3309 C whereas the water content increased with time at 15 °C during deacclimation in both Chambourcin and 3309 C rootstocks (Figure 4).

3.3. qRT-PCR analysis

3.3.1. *VvCBF2 (C-repeat binding factor 2)* and *VvCBF4 (C-repeat binding protein 4)*

In our research *CBF 2* gene expression was too low in bud samples of Chambourcin and 3309 C (one week at 5 °C

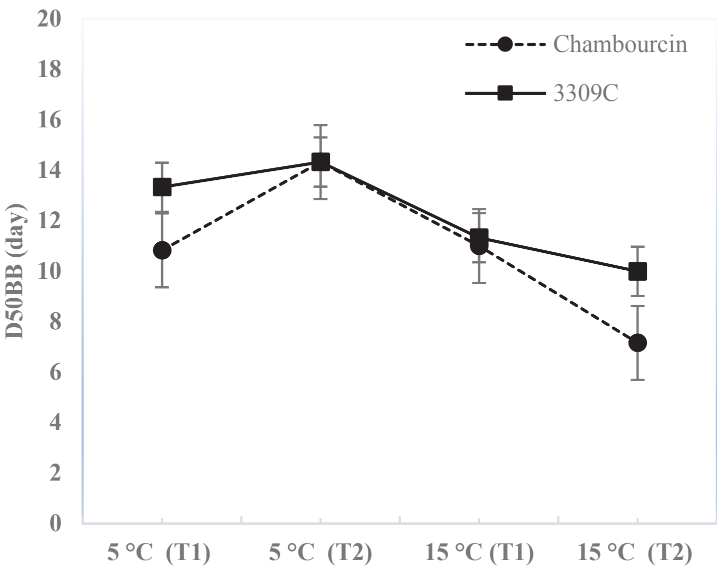


Figure 3. Bud dormancy (D50BB) curve of 3309 C and Chambourcin.

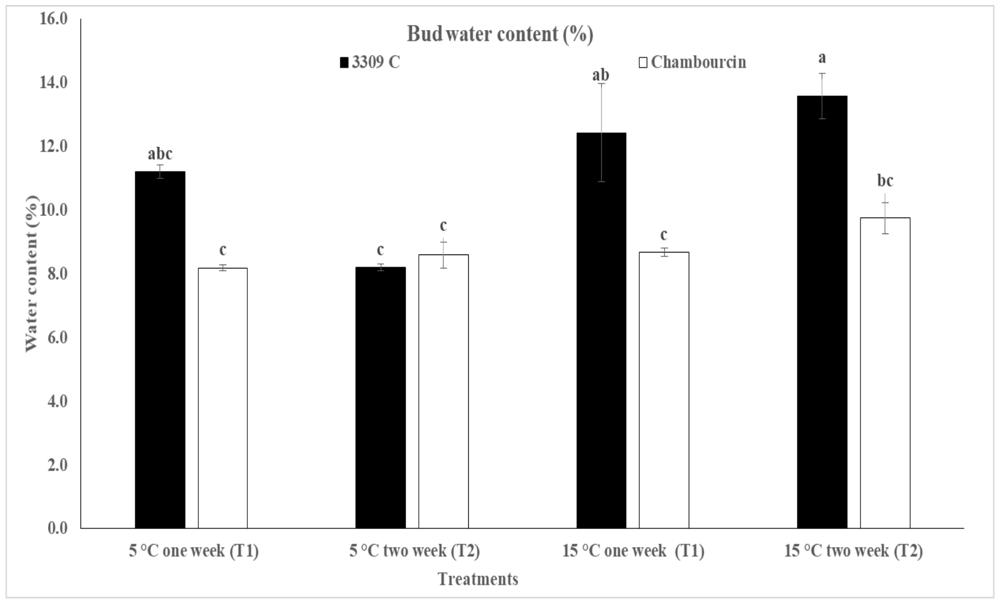


Figure 4. Mean bud water content (%) Chambourcin and 3309 C rootstock grape buds during acclimation (5 °C) and deacclimation (15 °C) (Different letters above bars indicate significantly different means (Tukey HSD, $p < 0.05$).

and 15 °C) from the growth chamber after acclimation and deacclimation. However, in the second group of samples (two weekss at 5 °C and 15 °C), *CBF 2* gene expression was high as 41.274 in rootstock 3309C buds. For *CBF 4* gene, the expression was low in the T1 and T2 groups of samples. According to our results, the expression of CBF genes was low during ecodormancy. Results of ANOVA analysis indicated that expression of *CBF2* and *CBF4* genes were significantly different for both times (first week and second week), genotypes (Chambourcin and 3309 C), temperature (5 °C and 15 °C). In addition, the interaction between time and genotype, time and temperature, genotype and temperature is also significant and shown in Figures 5 and 6, and Tables S1 and S2.

3.3.2. *NCED1 (9-cis-epoxycarotenoid dioxygenase)*

Between the two cultivars, the expression of *NCED1* varied according to time (one week, two weekss), genotype (Chambourcin and 3309 C), and temperature (5 °C and

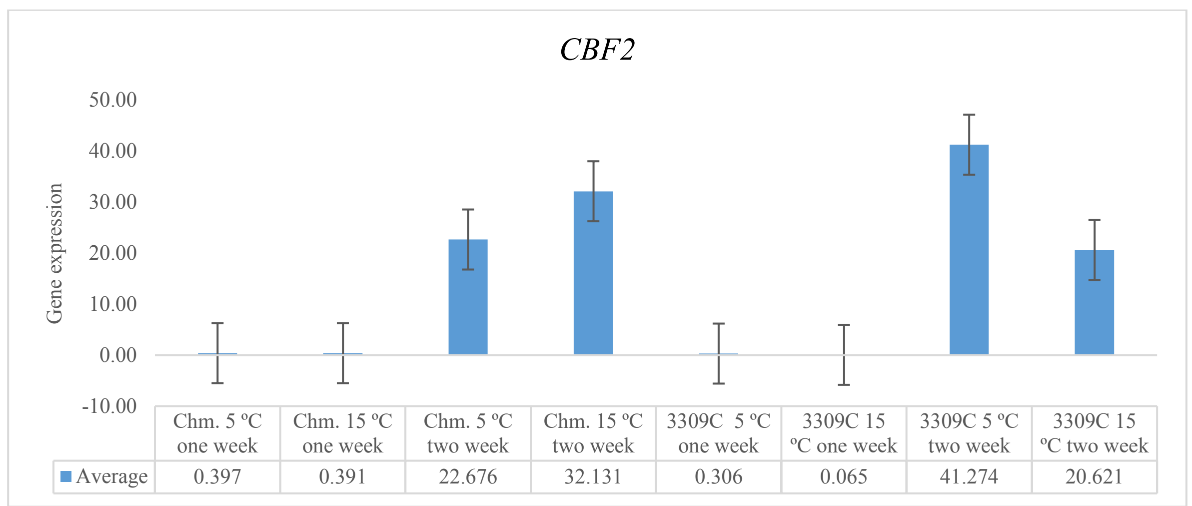


Figure 5. *VvCBF2* validated gene expression level during acclimation (5 °C) and deacclimation (15 °C) on Chambourcin and 3309 C (Bars indicate the differences that are statistically significant ($p < 0.05$ level). Data represent mean values from three independent biological experimental series. Error bars represent standard errors).

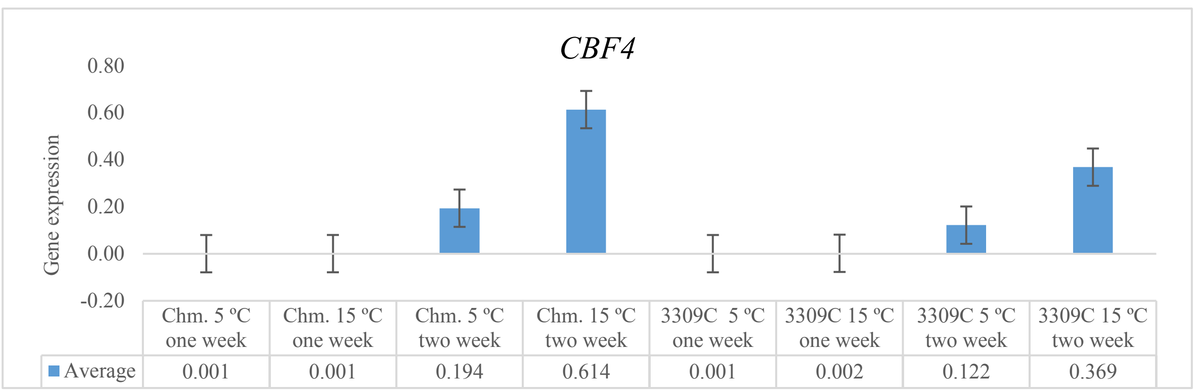


Figure 6. *VvCBF4* validated gene expression level during acclimation (5 °C) and deacclimation (15 °C) on Chambourcin and 3309 C (Bars indicate the differences that are statistically significant ($p < 0.05$ level). Data represent mean values from three independent biological experimental series. Error bars represent standard errors).

15 °C). In addition, the interaction between time and genotype, time and temperature, genotype and temperature was significant (Table S3). According to our results, "3309 C" had higher gene expression of *NCED1* compared to the "Chambourcin" during deacclimation. Samples in the first week (T1) showed the lowest gene expression level compared to the second week (T2). Rootstock 3309 C had a higher gene expression level than Chambourcin grape cultivar during acclimation and deacclimation time. Cold hardy 3309 C had the highest *NCED1* gene expression level during acclimation (5 °C) and deacclimation (15 C) (Figure 7).

3.3.3. *MYB14 (Myb-related transcription factor)*

The highest *MYB14* gene expression level was observed in Chambourcin at 5 °C in the second week (32.608). *MYB14* gene expression did not occur during T1 (one week at 5 °C and 15 °C). During acclimation at 5 °C, Chambourcin had the highest level of *MYB14* gene expression level (Figure 8). The results of ANOVA analysis for *MYB14* gene in both cultivars indicated that expression of this gene was significantly different at times (first week and second week), genotypes (Chambourcin and 3309 C), and temperature (5 °C and 15 °C). In addition, interaction between time and temperature as well as genotype and temperature were also

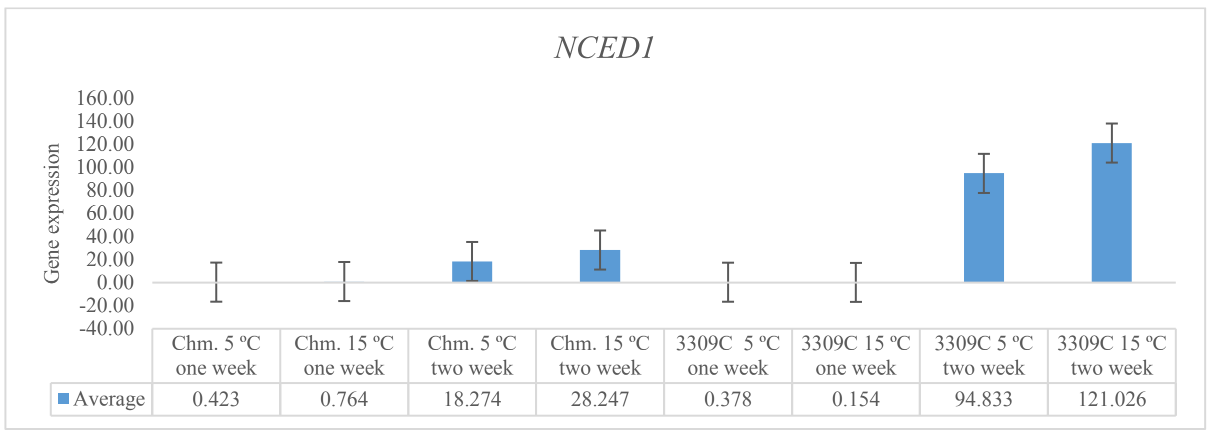


Figure 7. *NCED1* validated gene expression level during acclimation (5 °C) and deacclimation (15 °C) on Chambourcin and 3309 C (Bars indicate the differences that are statistically significant ($p< 0.05$ level). Data represent mean values from three independent biological experimental series. Error bars represent standard errors).

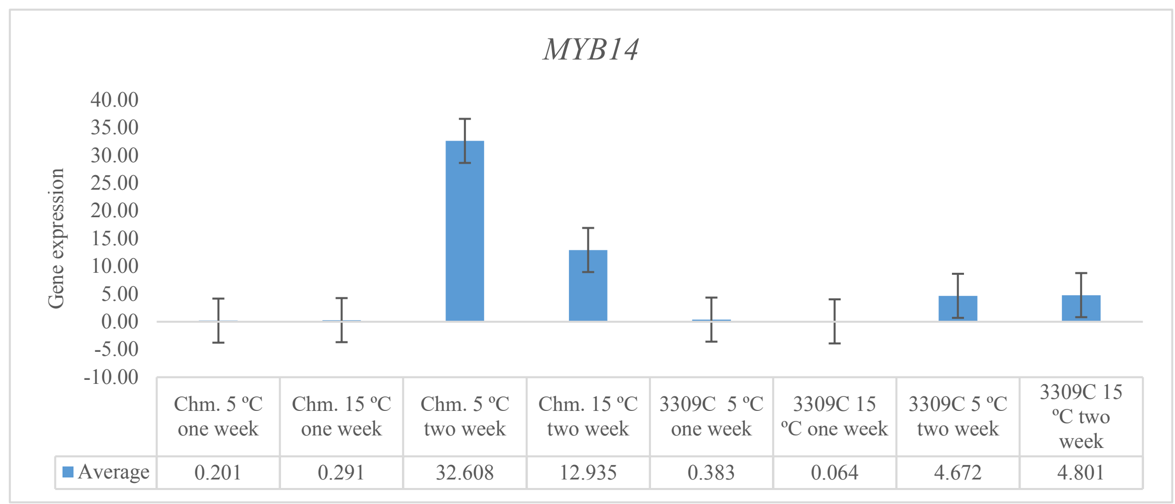


Figure 8. *MYB14* validated gene expression level during acclimation (5 °C) and deacclimation (15 °C) on Chambourcin and 3309 C (Bars indicate the differences that are statistically significant ($p < 0.05$ level). Data represent mean values from three independent biological experimental series. Error bars represent standard errors).

significant. However, time and genotype interaction is not statistically important (Table S4).

3.3.4. *PLD (Phospholipase D)*

According to our results, T1 sample's gene expression level was lower. However, T2 sample's *PLD* gene expression level was higher at rootstock 3309 C than Chambourcin grape cultivar during de-acclimation and e-acclimation. The highest gene expression level was at 3309 C rootstock as 86.404. For both rootstock and Chambourcin the expression was higher during deacclimation than acclimation (Figure 9). Results of ANOVA analysis for *PLD* gene in the studied cultivars indicated that expression of this gene was significantly different at different times (first week and second week), genotypes (Chambourcin and 3309 C), temperature (5 °C and 15 °C). In addition, interaction between time and temperature, genotype and temperature is also significant. However, time and genotype interaction is not statistically important (Table S5).

4. Discussion

Based on physiological and biochemical aspects along with genetic responses, the mechanism of grapevine bud dormancy was predominantly investigated, which suggested its association with various external factors, for instance, water status, photoperiodic cycle, temperature (Schrader et al., 2004) and bud dormancy (Min et al., 2017). The water content continues to decrease with lower

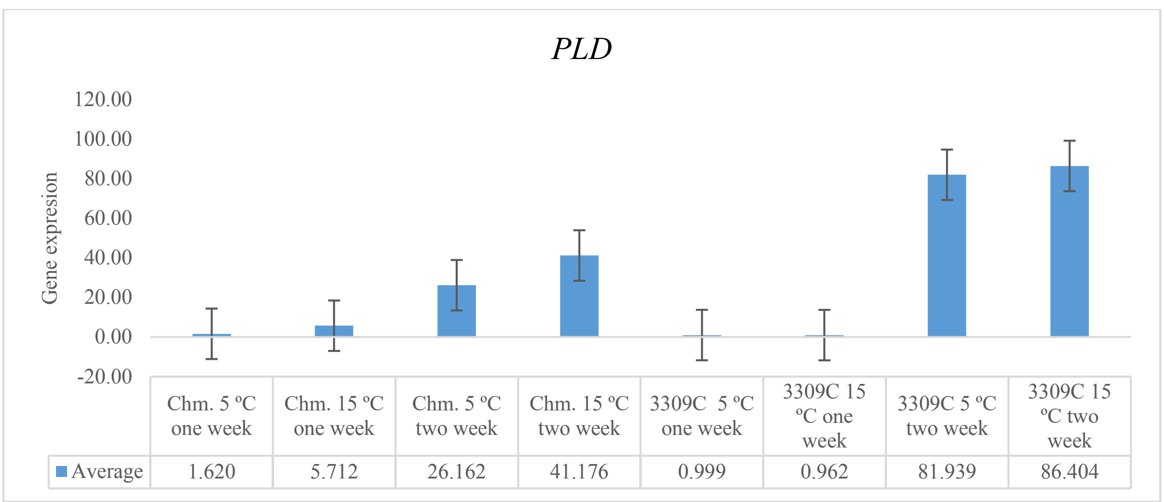


Figure 9. PLD validated gene expression level during acclimation (5 °C) and deacclimation (15 °C) on Chambourcin and 3309C. (Bars indicate the differences that are statistically significant ($p < 0.05$ level). Data represent mean values from three independent biological experimental series. Error bars represent standard errors).

temperatures in all buds and remains fairly constant until deacclimation begins in the spring, when temperatures begin to rise (Fennell, 2004). Our results also confirm this; the grape bud water content was lower during the acclimation process than during the deacclimation process.

D50BB ratio was higher in 3309 C rootstock than Chambourcin variety. At the same time, water content in dormant buds was found to be lower at 5 °C applications compared to 15 °C. In the controlled growth chamber, gene expressions during the acclimation and deacclimation were prominent in the second week applications. ABA-related *CBF2* and *NCED1* genes were high in 3309 C rootstocks at 5 °C and 15 °C. Similarly, *PLD* gene expression was higher in 3309C than in Chambourcin. *MYB14* gene was found high in Chambourcin grape variety at 5 °C (two week) application.

Well-characterized TF gene families in the *Vitis vinifera* were selected in this study. *CBF2* and *CBF4* transcripts were chosen in this study since previous studies confirmed their endurance with increased relative abundance during cold acclimation (Xiao et al. 2008; Tillet et al., 2012). ABA plays an important role in grapevine dormancy from the initiation of cold acclimation in the late summer through the release from dormancy in early spring (Wang et al., 2019). Different previous studies have demonstrated that *CBF* family genes act vital roles during the cold acclimation process for controlling important pathways related to this process (Xiao et al., 2008; Thomashow, 2010).

The increased expression of *Vitis riparia*'s *VvCBF* transcription factors was attributed to the species' superior capacity for cold adaptation, according to Karimi et al. (2015). qRT PCR tests showed that the *CBF2* gene was stimulated by low temperature but different extent in the more cold hardy *V. amurensis* and less cold hardy *V. vinifera*, supporting the hypothesis of Dong et al. (2013) that *CBFs* may not have an identical role. Rubio et al (2019a) indicated that interestingly, the expression of *VvCBF2* was inversely related to the expression of *VvCBF4* and *VvCBF6* after the treatments. *CBFs* have different expression profiles for each genotype which is similar to the investigation reported by Karimi et al. (2015). Close to the results of these studies, *CBF2* and *CBF4* had a varied expression for both cultivars. According to this study, *CBF4* regulation was shown to be extremely low whereas *CBF2* regulation was found to be high. Similar to the studies in the literature, *V. riparia* hybrid 3309 rootstock had a higher level of expression of *CBF2* during acclimation than *Vitis vinifera* cv. Chambourcin.

It has already known that ABA biosynthesis plays a vital role in the control of the dormancy process in vines. It was also observed that ABA synthetic enzyme *NCED* showed high regulation at the beginning of dormancy and low regulation during the exit from dormancy (Zheng et al., 2015; Shangguan et al., 2020). *NCED* enzyme activation for ABA synthesis is triggered during the dormancy induction and maintenance (Fennell et al., 2015; Wang et al., 2016; Wang et al., 2019).

In this study, cold hardy 3309 C had highest *NCED1* gene expression level during acclimation (5 °C) and deacclimation (15 °C). This outcome confirms the ABA metabolic synthesis continuation from acclimation to deacclimation (Wang, 2019). Previous studies, showed that the ABA levels increase as the grapevine buds enter endodormancy (Xiao et al., 2006; Xiao et al., 2008; Tillet et al., 2012). However recent studies reported that ABA plays an important role in the maintenance and release of

the bud from the endodormacy, as well as in its transition from the cold acclimation to the deacclimation (Rubio and Pérez, 2019a; Wang, 2019). Our results also confirm these findings, ABA related gene expressions level were high for *NCED1* and *CBF2* gene during cold acclimation and deacclimation. Recent research, however, indicated that ABA is crucial for release from endodormacy, as well as for its transition from cold acclimation to deacclimation (Rubio and Pérez, 2019a; Wang 2019). These findings, that *NCED1* and *CBF2* genes showed significant levels of ABA-related gene expression during cold acclimatization and deacclimation, are supported by the results of the current investigation. It is still unclear how ABA regulates metabolism and serves a variety of roles in plants (Rubio and Pérez 2019a). More research should focus on how ABA regulates stress responses and how environmental changes affect the dynamics of ABA metabolism (Pan et al., 2021).

MYB–type TFs from grapevine, regulate the stilbene biosynthetic pathway (Höll et al. 2013). *MYB14* regulates flavonoid metabolism; and in response to short days, flavonoid biosynthesis genes in grapevine buds were found to be up-regulated (Fennel et al., 2015). Biological process of *MYB14* gene is able to response to the cold and freezing conditions (Chen et al. 2013). In an experiment designed for studying several *R2R3-MYB*-type transcription factors of grapevine, it was revealed that *MYB14* and *MYB15* are capable of activating the promoters of resveratrol synthase/stilbene synthase (RS/StSy), and essential in stilbene production (Höll et al. 2013; Luo et al. 2020). According to differentially expressed genes (DEGs) profile, Kovaleski and Londo (2019) found the up-regulation of *MYB* genes during eco-dormancy. The results from this study showed that during acclimation at 5 °C (two week), Chambourcin had the highest level of *MYB14* gene expression level.

During the cold acclimatization process, it is noticeable that all the alterations occurred in gene expression are not directly associated with the cold tolerance (Wisniewsky et al., 2014).

PLD is a major gene family in higher plants significant for playing a vital role in the regulation of cellular processes, comprising root growth, root hair patterning, programmed cell death, abscisic acid signaling, freezing tolerance and other stress responses (Liu et al. 2010). Kovaleski and Londo (2019) reported that *PLD* phospholipase gene is up-regulated during ecodormancy process, which supports and is in agreement compatible with the findings of our present study. Li et al. (2004) found that overexpression of *PLD* results in higher freezing tolerance. In parallel with these results, compare to the Chambourcin higher gene expression was also found in 3309 C rootstock in this study during cold dormancy and release of dormancy.

5. Conclusions

Gene expression changes were investigated at two altered time phases during the cold acclimation and deacclimation process during controlled GC conditions. Differential expression analyses were implemented between consecutive time points which specifying the association of major transcriptional changes with ecodormancy. Comparing 3309C rootstock to Chambourcin, the *PLD* gene expression was higher (86.40) throughout acclimatization and deacclimation. At cold hardy 3309C, *NCED1* gene expression levels increased both throughout acclimation (94.83) and deacclimation (121.02). It is noteworthy to emphasize that all the variations in gene expression followed throughout the cold acclimatization process are interrelated with cold tolerance. The results of this investigation and current literature highlights indicate that ABA-related gene expressions were controlled during cold acclimatization and deacclimation.

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Conflict of interest

The authors declare no conflicts of interest in this work.

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Supplemantary File

Table S1. Analysis of variance for *CBF2* genes in Chambourcin and 3309 C at 5 °C and 15 °C.

Source	df	Mean Square	F	Sig.
time	1	5006.237	24.475	0
genotype	1	16.693	0.082	0.779
temperature	1	49.127	0.24	0.631
time * genotype	1	21.131	0.103	0.752
time * temperature	1	44.976	0.22	0.645
genotype * temperature	1	345.305	1.688	0.212
time * genotype * temperature	1	334.637	1.636	0.219
Error	16	204.547		
Total	24			
Corrected Total	23			

R Squared = .640 (Adjusted R Squared = .482)
Computed using alpha = .05

Table S2. Analysis of variance for *CBF4* genes in Chambourcin and 3309 C at 5 °C and 15 °C.

Source	df	Mean Square	F	Sig.
time	1	0.258	3.699	0.072
genotype	1	0.008	0.120	0.734
temperature	1	0.016	0.223	0.643
time * genotype	1	0.230	3.297	0.088
time * temperature	1	0.479	6.857	0.019
genotype * temperature	1	0.152	2.177	0.159
time * genotype * temperature	1	0.032	0.454	0.510
Error	16	0.070		
Total	24			
Corrected Total	23			

R Squared = .513 (Adjusted R Squared = .299)
Computed using alpha = .05

Table S3. Analysis of variance for *NCED1* gene in Chambourcin and 3309 C at 5 °C and 15 °C.

Source	df	Mean Square	F	Sig.
time	1	22648.075	150.879	0
genotype	1	9484.681	63.186	0
temperature	1	438.834	2.923	0.109
time * genotype	1	9632.566	64.171	0
time * temperature	1	433.206	2.886	0.111
genotype * temperature	1	81.681	0.544	0.473
time * genotype * temperature	1	93.92	0.626	0.442
Error	14	150.108		
Total	22			
Corrected Total	21			

R Squared = .944 (Adjusted R Squared = .916)
Computed using alpha = .05

Table S4. Analysis of variance for *MYB14* genes in Chambourcin and 3309 C at 5 °C and 15 °C.

Source	**df**	**Mean Square**	**F**	**Sig.**
time	1	1102.159	24.444	0
genotype	1	492.787	10.929	0.004
temperature	1	148.616	3.296	0.088
time * genotype	1	490.32	10.874	0.005
time * temperature	1	141.87	3.146	0.095
genotype * temperature	1	142.99	3.171	0.094
time * genotype * temperature	1	155.218	3.442	0.082
Error	16	45.09		
Total	24			
Corrected Total	23			

R Squared = .788 (Adjusted R Squared = .695) Computed using alpha = .05

Table S5. Analysis of variance for *PLD* genes in Chambourcin and 3309 C 5 °C and 15 °C.

Source	**df**	**Mean Square**	**F**	**Sig.**
time	1	19219.096	30.289	0.000
genotype	1	3429.650	5.405	0.034
temperature	1	207.682	0.327	0.575
time * genotype	1	4243.232	6.687	0.020
time * temperature	1	89.166	0.141	0.713
genotype * temperature	1	80.813	0.127	0.726
time * genotype * temperature	1	15.456	0.024	0.878
Error	16	634.527		
Total	24			
Corrected Total	23			

R Squared = .729 (Adjusted R Squared = .610) Computed using alpha = .05