Research Square

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Antioxidant Defense, Chlorophyll Fluorescence and *VvCBF4-VvNAC1* Genes Expression in Grapevine Cultivars (*Vitis Vinifera* L.) in Response to Cold Stress

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Research Article

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Abstract

Background: Cold stress is one of the limitative factors of different species of crops on the planet, causing significant damage to the Iranian agricultural industry every year. Grapes are the product of temperate warm zones and sensitive to early autumn cold and spring cold. The current study the effects of cold stress (+1 °C for 4, 8, and 16 hours) on three grapevine cultivars (Ghiziluzum, Khalili, and Perllete) were investigated.

Results: The results showed that cold stress caused significant changes in the antioxidant and biochemicals content in the studied cultivars. Furthermore, examining the chlorophyll fluorescence indices, cold stress caused a significant increase in minimal fluorescence (F0), a decrease in maximal fluorescence (Fm), and the maximum photochemical quantum yield of photosystem II (Fv/Fm) in all cultivars. According to the obtained results, among the three studied cultivars, 'Perllete' with the highest increase in proline content and the activity of antioxidant enzymes and also, having the lowest accumulation of malondialdehyde, hydrogen peroxide, electrolyte leakage, and F0 as well as less decrease in Fm and Fv/Fm had the higher tolerance to the cold stress than 'Ghiziluzum' and 'Khalili' cultivars. *VvCBF4* and *VvNAC1* genes expression was increased in all three cultivars at +1 °C at 8 hours and then decreased. The increase in *VvCBF4 and VvNAC1 genes* expression in 'Perllete' cultivar was higher than the other two cultivars.

Conclusion: 'Perllete' and 'Ghiziluzum' showed the highest tolerance to low temperature stress, respectively. 'Khalili' was sensitive to low temperature stress.

Background

Environmental stresses such as cold, salinity, and drought are the most important factors affecting the growth and production of crops. Given the growing population of the planet and the need for more food; producing plants with a high tolerance to the environmental stresses is of great importance. One of the factors that limit plants' survival and growth is cold stress which plays an important role in the ecological distribution of all plants [1]. In adaptation to cold stress, living organisms, especially plants, develop several mechanisms at the molecular, biochemical, and physiological levels to maintain their survival [2]. Cold stress is a direct result of the effects of low temperatures on cellular macromolecules that leads to slow metabolism and loss of membranes function [3]. The cell membrane is the outer living part of a plant cell. When the membrane is exposed to below-optimal temperatures, its status changes from a liquid phase to a gel one which interferes the membrane dynamics and function. The plasma membrane is a highly-organized system that plays important role is the relationship between the cell and the extracellular environment. In general, the result of cold stress is to lose the membrane health and leakage of solutes [4].

Imposing pressure on the cell wall, low temperature causes the expression of some genes in plants, resulting in membrane stability against cold damage and ultimately adaptation to cold [4]. Cold stress

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position, amount and function of enzymes, accumulation of

carbohydrates, amino acids, and soluble proteins in the cell [5]. The other consequence of low-temperature stress is the production of reactive oxygen species (ROSs). ROSs are toxic molecules capable of reacting with and damaging vital molecules such as proteins, nucleic acids, lipids, and carbohydrates. One of the most basic mechanisms for gaining tolerance to the environmental stresses is the elimination of reactive oxygen species (ROSs) [6]. Both enzymatic and non-enzymatic systems are effective in this process to deal with reactive oxygen species [3, 4]. Vineyards are affected by non-living stresses such as cold, drought, salinity, extreme temperatures, chemical toxicity and oxidative stress [1]. Although, the molecular basis of chilling acclimation is poorly understood, but the effect of some transcription factors involved in response to low temperature is well established [7].

A group of transcription factors called; CBF/DREB1 proteins was identified in *Arabidopsis* which regulates the expression of the genes for high resistance and adaptation to cold stress in plants [7, 8]. C-Repeat Binding Factors (*CBFs*) are transcription factors that have a vital role in gene regulation during cold acclimation in plant species [9]. Furthermore, the ectopic expression of *CBFs* from other plant species can increase the freezing tolerance of transgenic *Arabidopsis* [7]. In Grapevine, *CBF4* gene is often induced by cold treatment, while, *CBF1*, *CBF2* and *CBF3* respond better to drought [10]. The *NAC* transcription factor gene family is involved in regulating plant growth as well as the response to the biotic and abiotic stress. Previous research has described 8 types of *NAC* gene family members with different expression patterns under cold stress. Drought, salinity and cold stresses increase the expression of numerous *NAC* genes in *Arabidopsis* and other plants [11]. Expression of several *NAC* genes from *Arabidopsis* and wheat increases during various biotic stresses and in response to defense signaling pathway molecules such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), or ABA [12]. Studies have shown that *OsNAP* reduces H_2O_2 content, and many other *NAC* genes increase stress tolerance in various plant species by improving the capacity of the antioxidant system under drought stress [13]. Moreover, *OsNAP* reduces H_2O_2 content, and many other *NAC* genes increase stress tolerance in various plant species by raising up the capacity of the antioxidant system under drought conditions [14].

Applying cold stress; one can select grape cultivars that are more likely to tolerate the low-temperature conditions with monitoring the enzymatic and biochemical activities. The present study aimed to investigate the physiological and biochemical responses as well as *VvCBF4* and *VvNAC1*genes expression of three grape cultivars and their tolerance to the cold stress.

Results

Physiological and biochemical traits

Considering the interaction effect of stress × cultivar (Fig. 1); cold stress significantly increased proline accumulation in all grapevine cultivars compared to control; and, increasing the stress duration led to more proline accumulation in all treatments. In all cultivars, the lowest amount of proline belonged to the control treatment and the highest was belonged to 16-hour treatment. The highest amount of proline accumulation was in 'Perlette' cultivar (8.52 µmol/g of fresh leaf weight) with 16 hours of stress

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application and the lowest proline accumulation was traced in the control treatment of 'Perllete' (3.39 µmol/g of fresh leaf weight). The stronger effect of cold stress was on 'Perllete' which increased proline accumulation more than the control treatment and the lowest effect belonged to 'Ghiziluzum'. Mean comparisons (Fig. 1) showed that cold stress significantly changed the total protein content of the leaves, and the cultivars experienced different changes facing cold stress. In 'Perllete', 4 and 8 hours of cold stress increased the total protein content. But, increasing the treatment time up to 16 hours led to a significant decrease in the trait. In 'Khalili', cold treatments for 4 and 8 hours significantly increased the trait, but, 16 hours was not significantly different from 8 hours treatment. However, in 'Ghiziluzum'; increasing cold stress duration led to an upward increase in total protein content. Thus, the lowest total protein content belonged to the control treatment of 'Perllete' (0.0152 mg/fresh leaf weight) and the highest of protein content belonged to 'Ghiziluzum' with 16 hours of cold treatment (0.616 mg / fresh weight).

The means comparison (Fig. 1) shows that the cold stress caused a significant increase in malondialdehyde content in all studied cultivars compared to the control treatment. With all cultivars, the lowest accumulation of malondialdehyde belonged to the control treatment; and among the cultivars, the lowest value belonged to the control of 'Perllete' (1.309 nmol /g of fresh weight). The prolonged cold treatment time-course led to an increase in malondialdehyde content in all cultivars; so that, in all cultivars; the highest malondialdehyde content belonged to 16 hours of cold treatment. Therefore, the highest accumulation of malondialdehyde belonged to 16 hours of 'Khalili' (2.741 nmol/g of fresh weight). Mean comparisons revealed that, the APX activity in stressed plants was significantly different from control ones (Fig. 1). This difference was not uniform in the cultivars and different cultivars had different reactions to cold stress. Among the cultivars tested, the lowest ascorbate peroxidase activity belonged to the control treatment of 'Perllete' (0.209 units of enzyme per minute per gram of fresh leaf weight) and the highest activity was belonged to 16-hour treatment of 'Perllete' (2.916 units). Thus, the highest effect on the activity of this enzyme under the influence of cold stress was recorded for 'Perllete'.

Cold stress significantly increased the content of guaiacol peroxidase in all studied cultivars (Fig. 1). The lowest activity of this enzyme belonged to the control of 'Khalili' (2.09 units of enzyme per mg of fresh weight) and the highest amount belonged to 'Perllete' with 8 hours of cold stress (5.63 enzyme units/mg of fresh weight of leaves).Cold stress caused a significant change in the antioxidant capacity of all studied grape cultivars (Fig. 1). But, this change was not the same in all treatments and cultivars. In 'Ghiziluzum', the application of 4 hours of stress caused a significant increase in antioxidant activity and reached its maximum with 8 hours of cold treatment. However, by increasing the duration of treatment to 16 hours, there was a decrease in total antioxidant activity in this cultivar. Among the cultivars, the lowest amount of total antioxidant capacity belonged to the control treatment of 'Ghiziluzum' (5.553%) and the highest value belonged to 'Perllete' with 16 hours of cold treatment (8.309%) and 'Ghiziluzum' with 8 hours of treatment.

Cold stress increased the accumulation of H_2O_2 in the studied grape cultivars (Fig. 1). In 'Ghiziluzum',

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sed by the cold stress; and increasing the duration of

treatment raised the amount of H_2O_2 exponentially; so that, with 16 hours of cold treatment, H_2O_2 reached its maximum extent. The lowest accumulation of H_2O_2 belonged to the control treatment of 'Perllete' (6.34 mmol / l) and, the highest amount belonged to 'Ghiziluzum' with 12 hours of stress (41.44 mmol / l).Mean comparisons (Fig. 1) showed that the cold stress caused significant increase in electrolyte leakage in all three cultivars under cold treatments compared to the control. In all cultivars, the lowest percentage of EC belonged to the control treatment and the highest percentage belonged to 16 hours of cold treatment. Also, among the cultivars, the lowest percentage of EC belonged to the control treatment of 'Ghiziluzum' (3.66%) and the highest electrolyte leakage belonged to 'Khalili' with 16 hours of cold treatment (17.21%).

Correlation coefficient

As shown in table 4, the significant positive correlation value was observed among the traits at both 5% and 1% of probability levels in response to salinity stress. Considering, the significant positive relationship was recorded between the proline content with APX activity and electrolyte leakage. Furthermore, highly positive significant correlation was calculated between the total protein and the APX activity, H_2O_2 and electrolyte leakage. Moreover, the significant positive relationship was observed between GPX with electrolyte leakage. Furthermore, MDA and electrolyte leakage showed positive correlation (table 1).

Table 1 Coefficients of correlation values for the pairs of studied characters in three grapevine cultivars under cold stress

Y1 Y2 Y3 Y4 Y5 Y6 Y7 Y8

Y1 1

Y2 0.195 1

Y3 0.748** 0.562** 1

Y4 0.266 0.307 0.476** 1

y5 0.108 0.792** 0.406* 0.274 1

Y6 0.190 0.339* 0.043–0.090 0.287 1

Y7 0.021 0.147 0.101 0.294 0.112–0.323 1

Y8 0.536** 0.508** 0.409* 0.217 0.235 0.598** -0.181 1

**, * significant at $p \leq 0.01$ probability and significant at $p \leq 0.05$ probability, respectively. Y1 (Proline), Y2 (Protein), Y3 (APX), Y4 (Total Antioxidant), Y5 (H_2O_2), Y6 (MDA), Y7 (GPX), Y8 (Electrolyte Leakage).

Principal component and clustering analysis

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The principal component analysis (PCA) was exploited to distinguish plot of variation among the physiological attributes and to provide a more applicable understanding of the weight of each characteristic in the total variation. PCA analysis showed that the three factors or principal components explained 80.46% of total variations (Table 2). The first component (PC1) was the main and most efficient, responsible for about 45.58% of the total variance and proline, total soluble protein, APX, GPX and electrolyte leakage were the most significant variables (Table 2). Furthermore, the next two principal components; PC2 (18.13%) and PC3 (16.74%) accounted the rest of total variations (Table 2). In agreement with correlation analysis, the loading plot of characteristics in the PCA showed that the correlated attributes were located on the plot with close distances (Fig. 2A). Based on the dendrogram using cluster analysis (Fig. 2B), the traits were classified in a way similar to PCA and loading plot where proline and GPX, protein and H_2O_2 as well as MDA and electrolyte leakage were grouped under separate clusters, respectively.

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Table 2

Total Variance Explained (A), Component Matrix[a] (B)

Component	Initial Eigenvalues (A)		
	Total	% of Variance	Cumulative %
1	3.647	45.589	45.589
2	1.450	18.131	63.720
3	1.340	16.746	80.466
4	.736	9.194	89.660
5	.336	4.202	93.862
6	.297	3.717	97.579
7	.139	1.738	99.317
8	.055	.683	100.000
Extraction Method: Principal Component Analysis.			
	Component **(B)**		
	1	2	3
Y1	.754	− .599	.007
Y2	.746	.562	− .097
Y3	.820	− .206	− .379
Y4	.438	.063	− .643
Y5	.600	.653	− .176
Y6	.443	.303	.732
Y7	.728	− .460	.166
Y8	.755	− .023	.423
Extraction Method: Principal Component Analysis.			
a. 3 components extracted. Y1 (Proline), Y2 (Total soluble protein), Y3 (APX), Y4 (Total Antioxidant), Y5 (H_2O_2), Y6 (MDA), Y7 (GPX), Y8 (Electrolyte Leakage).			

Chlorophyll Fluorescence

One of the methods for estimating plant damage due to cold stress is chlorophyll fluorescence evaluation. Thus, cold tolerance in three grape cultivars was evaluated regarding chlorophyll

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mal fluorescence (F0) in all studied cultivars. In ‘Ghiziluzum’

and ‘Khalili’, 4 hours of cold stress significantly increased the minimal fluorescence; but, cold stress for 8 and 16 hours caused a decrease in this trait again. Cold stress in ‘Perllette’ did not have a significant effect on this trait and the treatments were not significantly different. The lowest minimal fluorescence belonged to the control treatment of ‘Khalili’ (0.953) and the highest value was belonged to ‘Ghiziluzum’ with 4 hours of cold treatment (1.43) (Fig. 3). The mean comparisons depicted (Fig. 3) that cold stress up to 4 hrs had a negative decreasing effect on maximal fluorescence (Fm) in all studied cultivars so that, the highest maximal fluorescence belonged to the control treatment of ‘Perllete’ (5.46) and the lowest value belonged to ‘Khalili’ with 4 hours of cold treatment (2.465). Cold stress in all three cultivars reduced the maximum photochemical quantum yield of photosystem II (Fv/Fm) compared to the control; so that in all cultivars, the highest value belonged to the control treatment. The highest amount of Fv/Fm belonged to the control treatment of ‘Perllette’ (0.739) and the lowest amount of Fv/Fm was recorded for ‘Khalili’ with 12 hours of cold treatment (0.481) (Fig. 3). At the low temperatures, the metabolism of leaf cells is severely inhibited mainly due to the effect of cold stress on the reduced carbon dioxide fixation or delayed photosynthetic cycles, the changes in the formation and distribution of sugars, and all these reduce the plant’s ability to recover from the stress damages.

VvCBF4 and *VvNAC1* gene expression

Comparison between the low temperature stress treatments compared to the control (22°C) showed that *VvCBF4 and VvNAC1* genes expression increased with adding up the stress time. At low temperature stress (8 hours at 1°C), the highest expression of *VvCBF4* gene (24.18 fold) and *VvNAC1* gene (32.81 fold) was observed in cultivar ‘Perllette’, which with increasing the low temperature stress time (16 hours at 1°C), the expression of *VvCBF4* gene (19.65 fold) and *VvNAC1* gene (14.22 fold) decreased in ‘Perllette’. The expression of *VvCBF4* gene for ‘Giziluzum’ and ‘Khalili’ cultivars was 17.21 and 14.65 fold for 8 hour at 1°C and for 16 hours at 1°C was 11.86 and 8.14 fold, respectively. The expression of *VvNAC1* gene for ‘Giziluzum’ and ‘Khalili’ cultivars was 26.16 and 20.45 fold for 8 hour at 1°C and for 16 hours at 1°C was 11.12 and 9.14 fold, respectively (Fig. 4).

Discussion

In cold-tolerant plants; proline is the predominant amino acid acting as a protective compound against cold and the elevated proline concentration in tissues is the mechanisms by which, to withstand cold stress. Moreover, the accumulation of this amino acid has been reported in many plants under cold stress due to the increased biosynthesis or a decreased degradation rate [2, 4]. Protecting the plant from osmotic changes, keeping the integrity of biological membranes, maintaining pH, protecting cellular enzymes, as well as storing energy for post-frost recovery are the major functions of proline in face of cold stress [18]. During cold stress, the total protein content increases which goes on to a certain extent and then decreases. These fluctuations can be interpreted that in the onset of stress, the plant begins to increase the expression of genes involved in the biosynthesis of defense enzymes to protect cellular structures to keep their normal activities. Therefore, by producing a sufficient amount of defense enzymes [illegible] crease the number of enzymes as a subset of total protein; and

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behind a sufficient period from the onset of stress, the conditions are under control by the plant cells [2–4]. Also, among the three cultivars studied, the lowest increase in malondialdehyde content compared to the control treatment was devoted to 'Perllete'. Malondialdehyde is one of the end products of membrane lipids peroxidation resulting from the reactive oxygen species activity. In other words, malondialdehyde levels are often used as an indicator of oxidative stress damage [19]. In the present study, cold stress increased the peroxidation of membrane lipids and as a result, increased the amount of malondialdehyde in the tissues of three grape cultivars. Commonly, the level of malondialdehyde in plant tissue is an indicator of stress-induced damage. Similar results on the effect of cold stress on malondialdehyde content were obtained in the previous studies with different grape cultivars [20]. Based on the results obtained in the present study, 'Perllette' can be introduced as the most tolerant among the tested cultivars, and 'Ghiziluzum' can be placed in the next rank in terms of cold tolerance. Plants show variety of morphological, biochemical, and physiological adaptations in response to stresses, including changes in the activities of certain enzymes such as ascorbate peroxidase. According to the literature, ascorbate peroxidase, as the most important antioxidant enzyme in plants; regenerates many free radicals especially hydrogen peroxide. The importance and role of this enzyme have been emphasized in many other plants especially in tangerine [21]. Higher APX activity was observed in *Jatropha macrocarpa* as a response to high H_2O_2, which improved cold stress tolerance, whereas reduced APX activity in *J. curcas* was linked with the increased sensitivity under cold stress conditions [22]. The results of the study conducted by Karimi Alvije et al., [1] also showed an increase in the content of guaiacol peroxidase in 7 different grapevine cultivars due to cold. They stated that placing grape seedlings at 4 ºC initially and significantly increased the content of this enzyme, but then stress caused decreasing pattern in its content. In cold-tolerant plants; the more efficient mechanisms enable them to protect themselves against the destructive effects of ROSs [4, 6]. This group of plants uses enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Glutathione Reductase (GR) as well as non-enzymatic compounds including Ascorbate, Tocopherol, Carotenoids and other compounds (including flavonoids, polyanols, mannitol) to gain the ability to reduce reactive oxygen species damage [1, 3, 6]. In plant cell, the AsA-GSH cycle is the major antioxidant defense pathway to detoxify H_2O_2, and redox homeostasis [21, 23]. Hydrogen peroxide and reactive oxygen radicals are produced under natural conditions in small amounts during the common metabolism in diverse organelles, including chloroplasts, mitochondria, and peroxisomes and in any places where there is an electron transport chain [5, 19]. 'Ghiziluzum' with its high H_2O_2 production was more sensitive to cold stress than 'Khalili' and 'Perllete' and had low cold tolerance. Numerous studies, including research on apples and pears, showed an increase in H_2O_2 accumulation under stress conditions [24]. Cell membrane is the first site of damage by the cold stress and the change in membrane state as a result of cold stress causes the membrane malfunction. So, measuring the electrolyte leakage of tissues is an acceptable criterion for evaluating plant resistance to cold stress [21, 22]. In the present study, 'Perllete' was more tolerant to the cold stress in terms of electrolyte leakage than 'Khalili' and 'Ghiziluzum'. The results obtained in this study were consistent with the findings on different grape cultivars [20], which reported the increased electrolyte leakage in response to the cold stress. Plant survival under environmental stresses, especially cold stress,

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nside plant cells, which ensure plant survival. These harmful

compounds can be ROS and compounds resulting from the oxidation of biological substances in cell metabolism. These compounds cause metabolic disorders due to intense electron demand. Antioxidant enzymes reduce cell damage by scavenging, ROS, and antioxidants are involved in reducing the oxidation of biomolecules, such as lipid peroxidation, by supplying the electrons needed [4, 6, 20].

When light is at a moderate level; its majority is employed in photochemical activities for photosynthesis and a small part of its energy is emitted as fluorescence known as basal or minimal fluorescence (F0) [25]. In the present study, the amount of F0 in all studied cultivars increased due to cold application. An increase in F0 indicates a damage to the photosystem II electron transfer chain due to a decrease in the capacity of quinone A (QA) and lack of its complete oxidation to a slow flow of electrons along the photosystem II pathway and the inactivation of photosystem II. A rise in F0 is associated with photoinhibitory damage but not with zeaxanthin retention [26]. The increased F0, due to cold stress, has been observed in plants such as basil (*Ocimum basilicum* L.) and lettuce (*Lactuca sativa*) [27]. The stress conditions causes structural changes in the pigments in photosystem II and the fluorescence function such as maximal fluorescence is changed, making it possible to use these factors as an indicator for estimating stress-induced damage to the plant photosynthetic system [25, 26]. The results showed that cold reduced the maximal fluorescence in all studied cultivars and this drop of Fm occurred at the maximum with applying 4 hours of cold stress. Researchers suggested that a decrease in Fm may be related to a decrease in the activity of the water-degrading enzyme complex as well as the electron transfer cycle in or around photosystem II [26, 27]. Research on tomatoes (*Solanum lycopersicum*) [27], also showed a decrease in Fm due to cold stress. Therefore, the measurement of Fv/Fm can be used as a successful method to determine the status of the photosynthetic apparatus and identify the degree of cold tolerance in plants [25]. By slowing down the insertion of protein D1 into the center of photosystem II, cold stress slows down the plants recover and causes membrane degradation and chlorophyll oxidation, thereby reduces the Fv/Fm ratio (maximum photochemical quantum yield of photosystem II under adaptive conditions to light). It is an estimate of the maximum photochemical quantum yield of photosystem II [28]. In many plant species, when the Fv/Fm ratio is about 0.7 to 0.8, it means that no stress has been applied on the plant. Therefore, values less than this amount indicate the effects of stress on plants [25–27]. In fact, chlorophyll fluorescence indicates a decrease in the initial health of the plant before the signs of deterioration appear, so that this trait indirectly indicates health (fluidity, stability, and cohesion) of photosynthetic membranes [25, 28]. Considering that high Fv/Fm indicates high cold resistance; among the three studied grapevine cultivars, 'Perllete' with the highest maximum photochemical quantum yield of photosystem II facing cold was more cold tolerant than the other two cultivars. Cold stresses increase F0 and decrease Fv/Fm, which indicates the discontinuity of light-absorbing pigments from the photosystem II complex, leading to a decrease in the quantum performance of photosystem II. The activity of the photosystem II is severely reduced or even stopped under cold conditions, and chloroplasts, stromal carbon metabolism, and photochemical reactions in the thylakoid lamella have been cited as primary sites of cold stress injury [25–28].

A study showed that drought, salinity and exogenous abscisic acid in plants caused *VaCBF4* expression. *bidopsis* increased the tolerance to cold, salinity and drought

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compared to control plants [29]. The expression of *VrCBF4* gene under stress conditions in different plant tissues continued for several days, which indicates the role of this gene in acclimation to stress conditions. Biological age did not affect *CBF4* expression, and transcription of this gene was observed in young and old leaves of *V. vinifera* and *V. riparia* for several days [10]. There are similar sequences in the promoter of two *CBF1* and *CBF4* genes, that protein product of *ICEr1* gene binds to that and induce this gene not only by cold but also by the other stresses [9]. In the present study, similar to some previous researches, *CBF4* gene expression accelerated immediately after cold exposure and decreased after 8 hours, but, is in contrast to the findings of other researchers who reported long-term accumulation of *CBF* genes [7, 10]. *VvNAC26* showed the greatest expression change in response to abiotic stress in the whole NAC family and acts as a *COR NAC* gene. *VvNAC18* induces freezing tolerance by increasing *DE NAC* genes, so *NAC* genes are expressed in response to cold stress and increase plant tolerance [11, 12]. *VvNAC1* may involve several signals, including developmental processes and responses to biotic and abiotic stresses, and can act as a novel node between different signaling pathways. Functional analysis has shown that *VvNAC1* has a positive role in abiotic stress tolerance [30]. NAC transcription factors increase stress tolerance in plants by modulating transcript levels of *CBFs* and their putative downstream such as *COR / ERD / RD* genes. One study showed that the function of *VaNAC17* in a *CBF*-dependent signaling pathway induced drought stress tolerance by modulating the expression of stress-responsive genes [14]. *NAC* transcription factors participate in various signaling pathways to against with cold stress, so the study of *NAC* transcription factors is a prerequisite for improving the effects of stress on plants. *NAC1* gene expression increases under cold stress by tolerant cultivars with producing signaling and increasing the expression of ROS inhibitory genes and also increases the response of *CBFs* genes, especially *CBF4*. CBFs cold-response proteins encode DNA-binding domains. These proteins bind to the DRE/CRT sequence and regulate cold-induced promoters.

Conclusion

In all three cultivars, the activities of guaiacol peroxidase and ascorbate peroxidase enzymes and total antioxidant capacity were increased in response to the cold exposure. Furthermore, cold stress exposure increased the accumulation of proline in leaf tissue in all cultivars. Electrolyte leakage and the concentrations of malondialdehyde and hydrogen peroxide, as the signs of cold damage, increased; but, this increase was different in various cultivars and cold levels. Cold stress caused damage to the photosynthetic system and therefore increased minimal fluorescence and, decreased the maximal fluorescence and maximum photochemical quantum yield of photosystem II. This damage was less in ‘Perllete’ than the other two cultivars. The increase in *VvCBF4* and *VvNAC1* genes expression in response to low temperature was more in ‘Perllete’ cultivar than the other two cultivars. And, this increase in *VvCBF4* and *VvNAC1* genes expression was highest in 8 hours at 1°C and then decreased. *VvNAC1* gene increased stress tolerance in three grape cultivar by modulating of *VvCBF4* gene expression and other effective genes.

Methods

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Plant materials

To investigate the effects of cold stress on three grapevine cultivars, ‘Khalili’, ‘Ghiziluzum’, and ‘Perllete’; their rooted cuttings were transferred to 5-liter plastic pots containing one-third of normal soil, perlite, and blown sand. Three cultivars ‘Khalili’, ‘Ghiziluzum’, and ‘Perllete’ are commonly cultivated in Iran. The homogeneous plant material (two years old rooted cuttings) were acquired from the nursery collection of the University of Maragheh. The plants were nourished with Hoagland's solution (Hogland & Arnon, 1950), pH was adjusted at 6.5. This experiment was conducted in the greenhouse of the Department of Horticultural Sciences, University of Maragheh, Iran, as a factorial based on CRD design with three grape cultivars (Biennial plants) at + 1°C for 4, 8, and 16 hours and at 22°C for the control treatment. The extended leaves were sampled after the completion of cold stress treatments to assay proline, total soluble protein, malondialdehyde, hydrogen peroxide and the activity of the antioxidant enzymes; catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX). Leaf samples were incubated in the liquid nitrogen and kept in the freezer (-80°C) until measurement.

Chlorophyll Fluorescence Indices

Chlorophyll fluorescence was measured by a fluorometer (model: PAM 2500-WALZ, Germany) from the last fifth leaves in the light. Minimal fluorescence (F0), maximal fluorescence (Fm), and maximum photochemical quantum yield of photosystem II (Fv/Fm) were assayed.

Proline content

Proline content was measured in wet plant tissue by Bates [15] method and the absorbance of the samples was recorded at 520 nm wavelength using a spectrophotometer. The control solution contained pure toluene.

Hydrogen Peroxide

0.2 g of the plant material was homogenized in 2 ml of 0.1% Tricloroacetic acid and centrifuged at 12000 g for 15 minutes. 0.5 ml supernatant was added to 0.5 ml of phosphate buffer (10 mmol, pH = 7) and 1 ml of Iodide potassium (1 mol). The samples absorbance was measured at 390 nm. Standard curves were established with the different concentrations of Hydrogen peroxide.

Malondialdehyde

0.2 g of the plant sample was homogenized in 2 ml of 20% Tricloroacetic acid containing 0.05% TBA. The samples later were incubated in 95 ºC for 30 minutes and they were transferred to the ice. The samples were then centrifuge at 10000 rpm for 10 minutes and the absorbance was measured at 532 and 600 nm. The extent of lipid peroxidation was obtained from the difference between the absorption wavelengths in the darkness coefficient of 155 mmol cm^{-1}.

Total Antioxidant Capacity

The antioxidant capacity of the extracts was calculated as the inhibition percentage of DPPH using the method of Chiou et al. [16].

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Antioxidant enzymes assay

For the extraction of Guaiacol peroxidase (GPX) and soluble proteins, 0.2 g of the sample was homogenized in liquid nitrogen. 2 ml phosphate buffer (pH = 7.5) containing, EDTA (0.5 mol) was added. The samples were incubated at 4 ºC for 15 minutes and were centrifuged at 15 rpm. Due to the instability and very low half-life of ascorbate peroxidase with ex-vivo conditions and for the keeping structure of the compound; we tried to use polyvinylpyrrolidone 5% and ascorbat (2 ml) to the respected enzyme solution.

Guaiacol peroxidase (GPX)

For GPX activity, the reaction mixture was containing 1 ml phosphate buffer (100mmol, pH = 7) along with EDTA (0.1 mmol), 1 mL guaiacol (15 mmol), 1 ml H_2O_2 (3 mmol) and 50 µL of the extracted enzyme solution. The reaction response was measured at 470 nm for 1 min. Enzymatic activity, based on the amount of tetraguaiacol, was obtained using a darkness coefficient of 26/6 mμ cm^{-1}.

Ascorbate peroxidase (APX)

APX was assayed as; the reaction mixture was containing 250 µL phosphate buffer (pH = 7) along with EDTA, 10 µL H_2O_2 (1 mmol), 250 µL sodium ascorbate (0.25 mmol) and 50 µL enzyme solution. The absorbance was measured at 290 nm for 1 min. Enzymatic activity was calculated using the darkness coefficient of 2.8 $mmol^{-1}$ cm^{-1}. The resulting number indicates the activity of Ascorbate Peroxidase based on micromoles of oxidized Ascorbate per minute.

Total Soluble Protein content

Reaction solution was contained 100 µL of enzyme solution, 200 µL of Bradford regent and 700 µL of deionizer water. 2 minutes after the complex formation; Bradford regent shows the highest integration with the amino acids. Absorbance was evaluated at 535 nm. Protein content of the samples was calculated based on standard curve obtained from the defined amounts of bovine serum albumin.

RNA extraction and DNA synthesis

Total RNAs were extracted and purified from the leaves following the method described by Tattersall et al. [17]. Only the extractions having an A260/A280 ratio of 1.8–2.0 and an A260/A230ratio > 2.0 were choosen for further analysis. The integrity of extracted RNAs was verified using 2% agarose gel electrophoresis followed by ethidium bromide staining. Oligo-dT, were used for first strand cDNA synthesis. The reaction mixture (Table 3) was prepared in a microtube on ice and was made up to 20µl using RNase-free water.

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Table 3 Reaction mixture for cDNA synthesis	
Reactive	Volume
Vivantis RT Enzyme Mix	0.5 µl
Buffer RT Enzyme	2 µl
Oligo RT Primer (50 µM)	0.5 µl
Random 6 mers (100 µM)	0.5 µl
dNTP	1 µl
DDW	11.5 µl
Total RNA (500 ng)	5 µl
Total	20 µl

RT-qPCR analysis

The RNA sequences of *VvCBF4* and *VvNAC1* genes were taken from NCBI (www.ncbi.nlm.nih.gov) and the forward and reverse primers were designed by Oligo 7 (Table 4). RT-qPCR analysis applied by an ABI StepOne Detection System (Applied Biosystems, USA), using the SYBR Green PCR Master Mix (TaKaRa, Toyoto, Japan). The reaction mixture (Table 5) was made up to 20µl total volume per sample. An initial denaturation step at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 60 s were performed. Following amplification, a melting curve analysis was performed to guarantee the absence of primer dimers and other nonspecific products. Relative quantification was executed by the comparative CT ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001). To quantify the transcript level, a standard curve (copy number as a function of Ct) was created by a 10×mass dilution series of each cDNA fragment. The exact copy number was presented by extrapolation of the Ct value for each cDNA on the standard curve and determined as copy number ng^{-1} of cDNA.

Table 4 Primer sequence of the genes *VvCBF4*, *VvNAC1* and *MDH* used for expression analysis in the present experiment

Primer Sequence (5'→3')

VvCBF4-F ACCCTCACCCGCTCGTATG

VvCBF4-R CCGCGTCTCCCGAAACTT

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VvNAC1-R TTCAGCTGGTTTTCCATGCTT

MDH-F ACAGCCTGCTTGCCAGTTAC

MDH-R CCAAATCTTCTTGCGGGTC

Table 5
The composition of reaction mixture for RT-PCR

Reactive	**Volume**
RT reaction solution (cDNA)	**2 µl**
Primer F	0.4 µl
Primer R	0.4 µl
Power SYBR Green PCR Master Mix	10 µl
DDW	7.2 µl
Total	20 µl

Statistical Analyses

The present study was conducted based on a factorial experiment in a completely randomized design. Data analysis was conducted by SAS software (version 9.1.3) and data means were compared using Duncan's multiple range test at 5% probability level. Tables and graphs were drawn using Office software (2016).

Abbreviations

F0: Minimal fluorescence**;** Fm: Maximal fluorescence**;** Fv/Fm: Maximum photochemical quantum yield of photosystem II; *VvCBF4*: *Vitis vinfera* C-repeat binding factor 4; *VvNAC1*: *Vitis vinfera* Nucleus Accumbens Associated 1

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Study conception and design, performed experiments, drafting of manuscript done by M.A.A.; analysis of data, improvement of the manuscript done by M.B.H. and M.AA. All authors reviewed the manuscript.

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Figures

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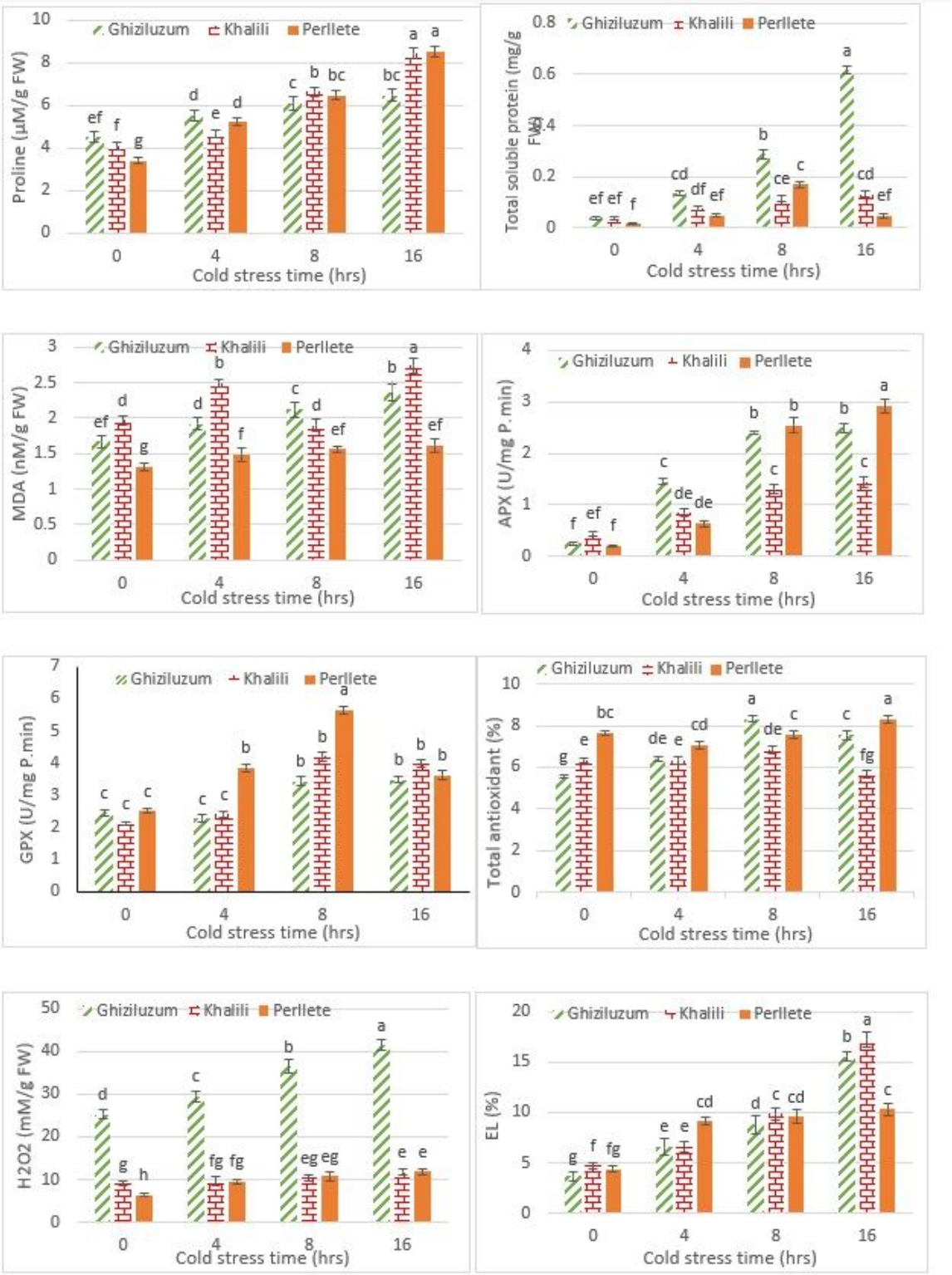

Figure 1

The effects of cold stress time-course and cultivar on the total soluble protein, proline content, malondialdehyde MDA), Ascorbate peroxidase (APX), guaiacol peroxidase (GPX), total antioxidant, H2O2 and Electrolyte Leakage (EL) of three grapevine cultivars. Similar letters show no meaningful difference at 5% probability level by Duncan's Multiple Range Test. Data are mean±SD (n=3 replicates).

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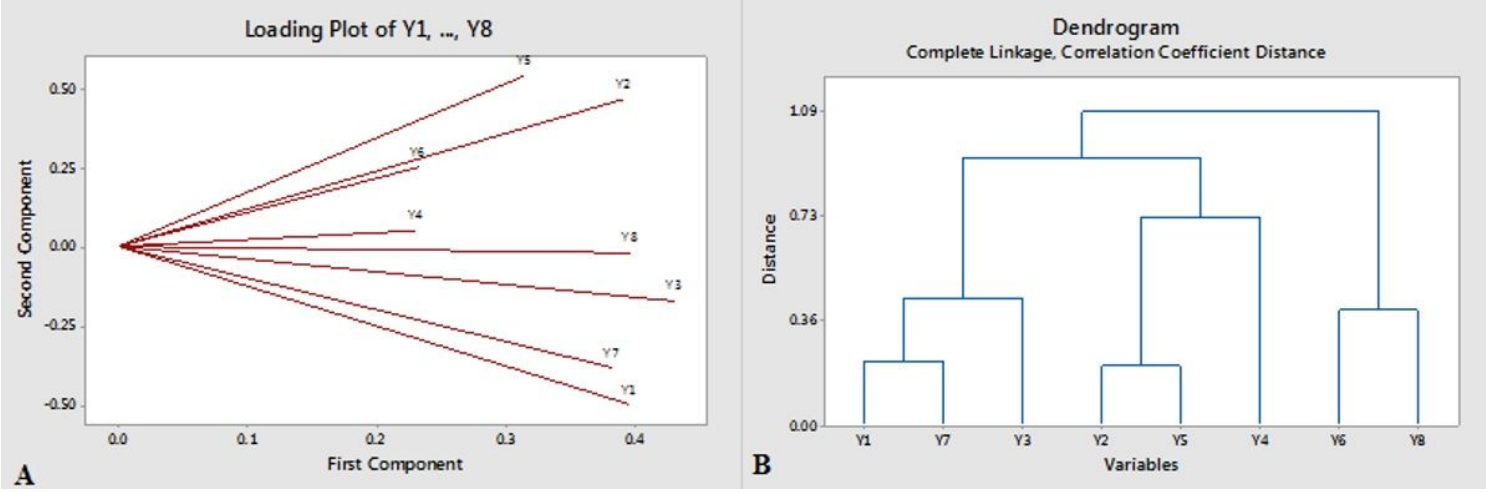

Figure 2

Bi-plot demonstration of 8 traits of 3 grapevine cultivars under cold stress based upon principal components (A). Dendrogram of 8 traits of 3 cold stress faced grapevine cultivars based upon principal components (B). Y1 (Proline), Y2 (Protein), Y3 (APX), Y4 (Total Antioxidant), Y5 (H2O2), Y6 (MDA), Y7 (GPX), Y8 (Electrolyte Leakage).

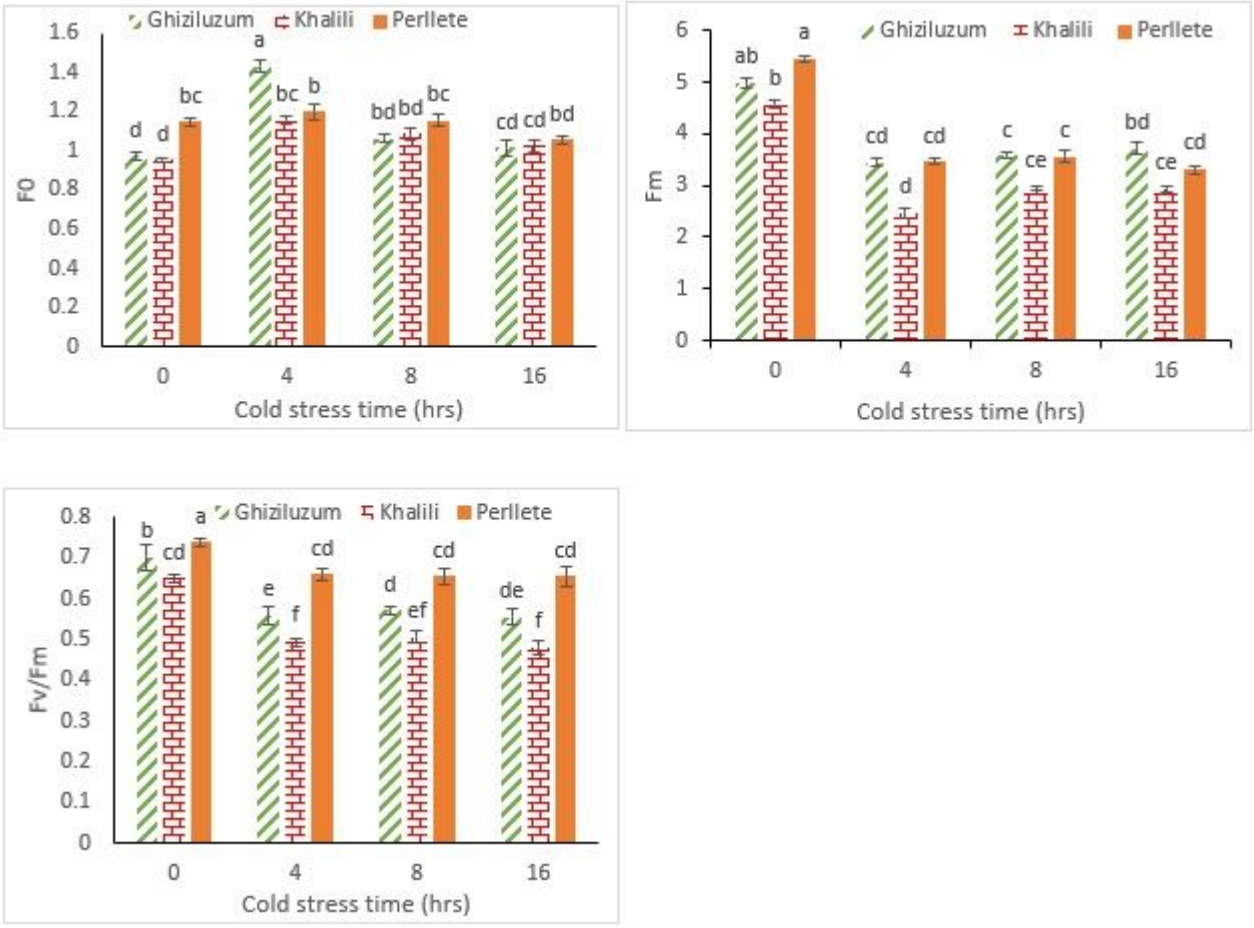

Figure 3

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The effects of cold stress time-course on F0 (minimal fluorescence), the Fm (maximal fluorescence) and Fv/Fm (maximum photochemical quantum yield of photosystem II) of three grapevine cultivars. Similar letters indicate no significant difference at 5% probability level by Duncan's Multiple Range Test. Data are mean±SD (n=3 replicates).

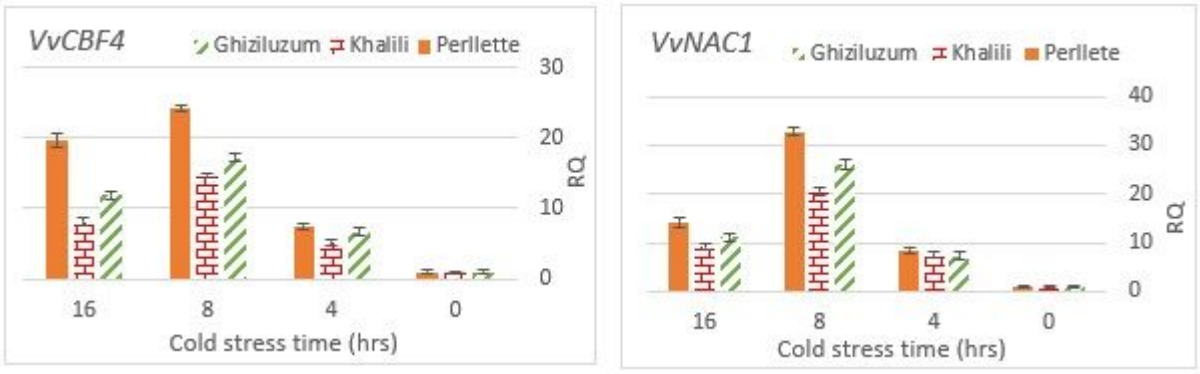

Figure 4

The effects of cold stress time-course on VvCBF4 and VvNAC1 gene expression of three grapevine cultivars. Data are mean±SD (n=3 replicates).

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