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Different response of GTP cyclohydrolase I gene from grape under abiotic stresses

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Abstract
Folates are a vital necessity for the retention of normal cellular activity. In contrast to humans, other organisms including plants gain folate coenzymes via *de novo* synthesis. GTP cyclohydrolase I (gtpch I, EC 3.5.4.16) catalyzes the first step of the biosynthesis of tetrahydrofolate (FH_4) in plants by the conversion of GTP to dihydroneopterin triphosphate and formic acid. In this research, the expression pattern of the Vv*gtpch* I gene was assayed in different organs of the grape by the semi-quantitative RT-PCR. The analyses demonstrated that the Vv*gtpch I* gene was expressed in all grape organs. The highest amounts of expression were obtained in berry and leaf, whereas the lowest amount of Vv*gtpch* I transcript was related to the cluster. The response of Vv*gtpch* I gene to abiotic stresses was also investigated under the alkali and cold stresses by the semi-quantitative RT-PCR. Under the alkali stress, the transcript level of Vv*gtpch* I gene decreased considerably. Similar to the alkali stress, the transcript level of Vv*gtpch* I gene decreased under cold stress as well. To analyze the Vv*gtpch* I gene expression under oxidative stress, different treatments were applied such as chemical inducers, heavy metals, and plant growth regulators to trigger the production of reactive oxygen species. The Vv*gtpch* I showed a strong increase and a moderate increase in the transcript amount with Cu^{2+} and H_2O_2, respectively. Whereas, its transcript level was relatively down-regulated by the heavy metals and hormonal treatments, and almost disappeared by diamide.

Keywords: folate, gene expression, oxidative stress, reactive oxygen species, abiotic stress

Abbreviations: ABA: abscisic acid; EST: expressed sequence tag; FH_4: tetrahydrofolate; gtpch I: GTP cyclohydrolase I; PGR: plant growth regulator; ROS: reactive oxygen species; RT-PCR: reverse transcription-PCR; SA: salicylic acid.

Introduction

GTP cyclohydrolase I (gtpch I, EC 3.5.4.16) is involved in the conversion of GTP to dihydroneopterin triphosphate and formic acid via a complicated series of reactions. This reaction is the first step in the biosynthesis of tetrahydrofolate (FH_4) and tetrahydrobiopterin (BH_4) in plants, and some microorganisms, and mammals, respectively (Blau and van Spronsen 2014). Folates belong to an extensive family of polyglutamates known as vital cofactors for biosynthesis of purines, pyrimidines, pantothenate, thymidylate, and in the metabolism of some amino acids, such as methionine, serine, and glycine (Blancquaert *et al*. 2014). Deficiency of folate in the diet

results in neural tube defects in infants (including spina bifida and anencephaly) (Imbard *et al.* 2013), increased risk of vascular disease, some cancers, and cerebral folate deficiency syndromes in childhood (Aune *et al.* 2011; Perez-Duenas *et al.* 2011). However, folic acid fortification of grain products has caused a considerable reduction in the occurrence of neural tube defects, some childhood cancers, and stroke (Strobbe and van Der Straeten 2017).

Humans cannot make folate in their bodies and must get it from food sources including crops, animal-based foods, or nutritional supplements. The staple crops such as wheat (10 to 91 µg/100 g FW), maize (33 to 129 µg/100 g FW), rice (11 to 111 µg/100 g FW), and soybean (344.06–685.81 µg/100 g FW) contain high levels of folates (Ashokkumar *et al.* 2018; Liang *et al.* 2020; Shahid *et al.* 2020; Agyenim-Boateng *et al.* 2023a). However, folate intake in humans often is lower than the prescribed rates of 400 µg/day for adults and 600 µg/day for pregnant women (Agyenim-Boateng *et al.* 2023b). Biofortification through conventional breeding and/or plant genetic engineering is a sustainable approach to enriching crops with folates (Ashokkumar *et al.* 2020).

The Vv*gtpch* I gene with chromosomal position 1 was submitted in NCBI GenBank database under RefSeq accession number XM_002269229. The Vv*gtpch* I genomic sequence with 4,964 bp length contains two exons and an intron. Also, its ORF is 1,338 nucleotides in length encoding a protein with 445 amino acid residues. The computed molecular mass and the predicted isoelectric point of the deduced polypeptide are 48.65 kDa and 6.43, respectively. The Vv*gtpch* I gene expression has been analyzed in various plants under different abiotic stress conditions. Changsong and Diqiu (2010) assayed the transcript expression pattern in mature pollen of *Arabidopsis* using Affymetrix GeneChips containing~24,000 genes. They showed that *gtpch* I gene is down-regulated in the mature pollen of *Arabidopsis* under cold stress. Moreover, analysis of folates in seedlings, inflorescence, and dry seeds of *Arabidopsis* indicated stable levels in the wild-type, mutant, and overexpressor line, upon heat and drought stress and normal conditions, indicating no induction of FH_4 biosynthesis genes, especially *gtpch* I gene (Navarrete *et al.* 2012). Furthermore, it has been reported that *gtpch* I gene from the grape was differently induced under different abiotic stress conditions. Its transcript level decreased significantly under drought, salt, and heat whereas it was increased under heavy metal treatments (Eslami-Bojnourdi *et al.* 2017).

The *gtpch* I has been a suitable gene for metabolic engineering because it is imagined to be the rate-limiting step controlling flux into the folate pathway (Wakeel *et al.* 2018). In rice

(*Oryza sativa*), folate biofortification of seeds obtained 100 times above wild type via overexpression of two *Arabidopsis thaliana* genes coding for *gtpch* I and aminodeoxychorismate synthase (Storozhenko *et al*. 2007). Nunes *et al*. (2009) produced transgenic lettuce lines containing a synthetic codon-optimized *gtpch* I gene based on the native red jungle fowl (*Gallus gallus*) gene. Immunoblotting analyses affirmed the presence of the *gtpch* I in the transgenic lines. Another example of plant genetic engineering was reported in tomato fruit in which the aminodeoxychorismate synthase gene was overexpressed through the gene from *Arabidopsis*. Compared with the wild-type, the transgenic fruit contained an average of 19-fold more p-ABA, without any enhancement in the folate amount (Diaz de la Garza *et al*. 2007). In contrast, Ramirez Rivera *et al*. (2016) introduced *gtpch* I gene from *Arabidopsis* into three common bean cultivars by particle bombardment and enhanced the folate levels in common bean via engineering the pteridine branch required for their biosynthesis. Recently, De Lepeleire *et al*. (2018) have indicated that the introduction of downstream genes in mitochondrial folate biosynthesis (HPPK/DHPS and/or FPGS) results in the enhancement of folate to appropriate levels (12-fold) and ensures folate stability upon long-term storage of potato (*Solanum tuberosum*) tubers.

Here, we analyzed the transcript level of the Vv*gtpch* I gene from the grape (*Vitis vinifera* L. cv. Askari) under non-stress and stress conditions by the semi-quantitative RT-PCR. The aim was to show that the Vv*gtpch* I gene differentially expresses in different organs of grape. Moreover, we wanted to reveal that Vv*gtpch* I gene is differentially induced upon abiotic/oxidative stress conditions.

Materials and Methods
Plant materials
Different organs of grape (*V. vinifera* L. cv. Askari) in clouding berries, leaves, petioles, clusters, roots, and seeds were prepared from the Grape Research Station, Takistan, Qazvin, Iran, during the 2018 field season. After immediate freezing in liquid nitrogen, all samples were stored at -80 ℃ until use. By gently breaking the berries in liquid nitrogen, seeds were separated from the berries at the veraison stage, then pericarp and seed portions were separately stored at -80 ℃.

Total RNA extraction and first-strand cDNA synthesis
Total RNA was extracted from different grape organs as described by Heidari-Japelaghi *et al*. (2011). First-strand cDNA was synthesized with 2 µg of total RNA treated with DNase I (Fermentas) as a template and 1 µg Oligo (dT)$_{18}$ primer (Qiagen) for 5 min at 70 ℃.

After incubation of the reaction mixture with RevertAid^{TM} M-MuLV Reverse Transcriptase (200 u/µl, Fermentas) for 60 min at 42 ℃, the reaction was stopped by heating the mixture at 70 ℃ for 10 min.

Abiotic stress treatments

To study the response of Vv*gtpch* I gene to various abiotic stresses, the cuttings were treated with abiotic stimuli in three independent replicates. For the alkali stress, the cuttings were treated with various concentrations of NaHCO$_3$, including 25, 50, 75, 100, 125, and 150 mM (Gong *et al.* 2013). The cuttings were gently exposed to different concentrations of NaHCO$_3$ during 5–7 days and then the youngest fully expanded leaves were harvested after almost seven days. Control and alkali-treated cuttings were incubated under a 16 h light period with a photon fluence rate of 120 µM quanta m^{-2} s^{-1} at 21 °C and 50% humidity in a greenhouse. Cold stress was also accomplished by exposing the cuttings to a temperature of 4 °C for 6, 12, 18, 24, 48, and 72 h under a 16 h light period, a photon fluence rate of 120 µM quanta m^{-2} s^{-1}, and 50% humidity in a growth chamber (Grouc, Iran).

Oxidative stress treatments

To test whether the steady-state transcript levels may respond to oxidative stress, varied treatments were applied including chemical inducers, heavy metals, and PGRs to induce the production of reactive oxygen species. The effectors were used to leaf slices through incubation in the effector solution after infiltration to ensure quick and homogenous application. Three to four youngest fully expanded leaves were removed from one-year-old cuttings and cut into 1 cm diameter leaf slices. After vacuum infiltration with deionized distilled water, the leaf slices were suspended in effector solutions (pH 5-6) including 10 mM H$_2$O$_2$, 1mM diamide, 100 µM CuSO$_4$, 100 µM CoCl$_2$, 100 µM CdCl$_2$,100 µM AlCl$_3$, 100 µM abscisic acid (ABA), and 100 µM salicylic acid (SA). The leaf slices were incubated at 21 °C and a photon fluence rate of 120 µmol quanta m^{-2} s^{-1} for 4 h.

Semi-quantitative RT-PCR

The semi-quantitative RT-PCR amplifications were performed with 100 ng of the reverse transcription product in a final volume of 20 µl containing specific primers (10 pmol) for the coding sequence of Vv*gtpch* I gene (Table 1). In parallel, the grape actin gene (Vv*act*) as an internal control was also amplified in all samples. PCR reactions were carried out in a programmable thermal cycler with the following temperature parameters: the initial denaturation for 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 58 °C, 1 min at 72 °C, and the final extension for 5 min

at 72 °C. A reaction without a template was used as a negative control for each set of RT-PCR amplifications and reactions were carried out in triplicates. RT-PCR products were detected by the agarose gel electrophoresis and quantified using ImageJ software (W.S. Rasband; 1997–2007; National Institutes of Health; http://rsb.info.nih.gov/ij). The normalization of signal intensities was accomplished concerning Vv*act* gene from the same sample.

Table 1. Nucleotide sequence of oligonucleotide primers used for semi-quantitative RT-PCR designed by Oligo7 software.

Primer name	Sequence (5'-3')	Melting temperature (ºC)	Size of amplicon (bp)
Semi-quantitative RT-PCR			
Vvgtpch IF	ATGGGCGTCTTGGACGAGG	61.5	1,338
Vvgtpch IR	TCAAGAAGTTGGAGTGTTTTGAA	58.6	
VvactF	GTTAGCAACTGGGATGATATGG	66.8	530
VvactR	AGCACCAATCGTGATGACTTG	69.6	

Statistical analysis

The data were evaluated statistically by SPSS ver. 16 (SPSS Inc., Chicago, IL, USA), and comparisons between the treatment means were made by the Duncan multiple range test at the probability level of $p \leq 0.05$. The data were expressed as mean ± standard deviation of three biological replicates.

Results and Discussion

Expression profile of Vvgtpch I in different organs

The number of expressed sequence tags (ESTs) coding for Vv*gtpch* I in various organs was accounted for among the whole grape ESTs recorded on the NCBI GenBank database. Around 13 ESTs were detected for Vv*gtpch* I in different grape organs including flower (6 hits), leaf (3 hits), root (3 hits), and berry (1 hit). However, no EST coding for Vv*gtpch* I was found in clusters, petioles, and seeds in the NCBI GenBank database. Based on the number of identified ESTs, the Vv*gtpch* I gene probably appeared to be transcribed at a low level in different organs. Similarly, analysis of RNA-Seq mRNA baseline using the Expression Atlas Database revealed that the Vv*gtpch* I gene may express at medium or low levels in different cultivars or in various organs at different developmental stages (Figure 1a).

The analyses based on semi-quantitative RT-PCR demonstrated that the Vv*gtpch* I gene was expressed in all grape organs. The highest levels of expression were observed in berries and leaves, whereas the lowest amount of Vv*gtpch* I transcript was related to the clusters. Petioles, roots, and seed organs harbored almost similar transcript levels (Figure 1b). Waller *et al*. (2010) reported that changes in

transcript abundance of *gtpch* I gene during fruit development of tomato were maximally expressed at the mature green stage, and declined at the breaker, red, and red ripe stages. Similarly, transcripts for gtpch I were not detectable after the early phases of fruit ripening in tomato. The mRNA level of gtpch I was highest in unripe (mature green and breaker stage) fruits. As the ripening progressed, the mRNA level declined aggressively and was scarcely detectable when the fruit reached full color and was softening
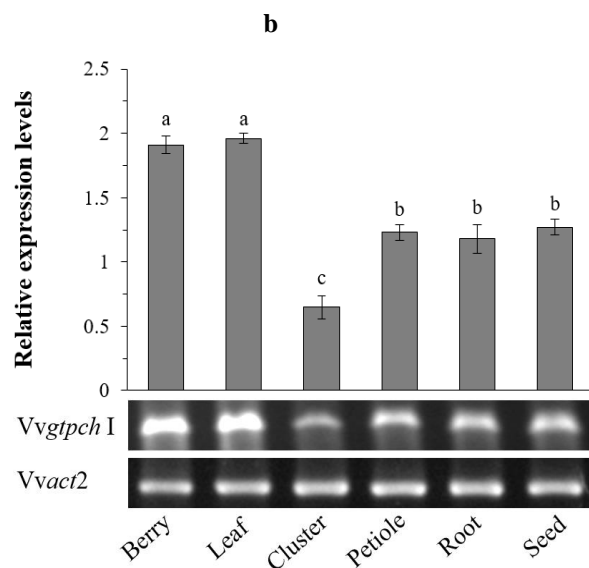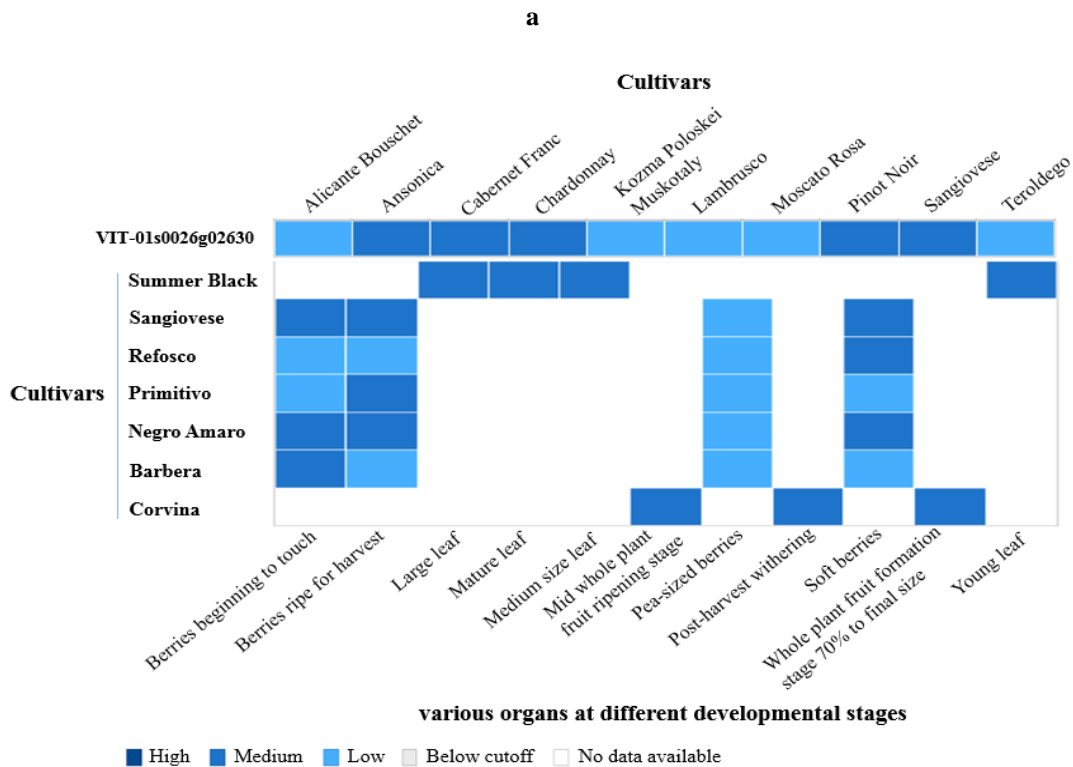

Figure 1. Expression of the Vv*gtpch* I gene in different grape organs. (a) Analysis of RNA-Seq mRNA baseline of the Vv*gtpch* I gene in different cultivars or various organs at different developmental stages using the Expression Atlas Database, (b) Analysis of expression pattern of the Vv*gtpch* I gene in different grape organs by the quantitative real-time-PCR and the semi-quantitative RT-PCR.

(red-ripe stage) (Basset *et al.* 2002). A differential expression profile of *gtpch* I mRNA took place overall seed development, whereas folate accumulation was reduced. The *gtpch* I transcripts were present in the embryo and maternal layers but not the endosperm (McIntosh *et al.* 2008).

Expression analysis of Vvgtpch I gene under abiotic stresses

Under abiotic stress conditions, plants have extended different mechanisms via a combination of metabolic, physiological, and morphological changes. These adaptive changes depend mostly on alterations in gene expression (Kamal *et al.* 2010). The salinity and alkalinity as two major abiotic stresses cause both hyperionic and hyperosmotic conditions, which finally lead to plant death. The tolerance to salinity and alkalinity is controlled by various genes involved in different molecular processes, such as selective ion uptake (Shi *et al.* 2000), accumulation of osmoprotectants and antioxidants (Blokhina *et al.* 2003), and expression of transcription factors (Baena-Gonzalez *et al.* 2007). Cold stress negatively influences the growth and development of plants via direct prohibition of metabolic reactions and, indirectly, via cold-induced osmotic, and oxidative stresses (Chinnusamy *et al.* 2007). The up-regulation of several genes

in response to cold stress, including cold-responsive genes encoding molecular chaperones, and several signal transduction and regulatory proteins was also reported (Mahajan and Tuteja 2005). Moreover, by transcriptomic analyses, the expression of *gtpch* I and II genes has been affected by biotic and abiotic stresses in pepper (*Capsicum annuum*) (Kim *et al.* 2019) and in wheat (Gupta *et al.* 2016; Diaz *et al.* 2019), respectively.

Under the alkali stress, the transcript level of Vv*gtpch* I gene decreased considerably. The amount of transcript of Vv*gtpch* I gene gradually increased to 100 mM $NaHCO_3$ and then highly decreased upon very severe stress conditions (100 mM $NaHCO_3$) (Figure 2a). Similar to the alkali stress, the transcript level of Vv*gtpch* I gene decreased under cold stress. The amount of transcript of Vv*gtpch* I gene gradually increased to 18 h and then there was a highly dramatic decrease to 72 h of cold treatment. When treated plants were incubated for one week at 21 °C, the transcript level of Vv*gtpch* I gene increased to the control level (Figure 2b). We have already shown that the transcript level of Vv*gtpch* I gene decreased significantly under abiotic stress conditions such as drought, salt, and heat (Eslami-Bojnourdi *et al.* 2017). In addition, similar to our results, a down-regulation of *gtpch* I gene has also been found in the mature pollen of
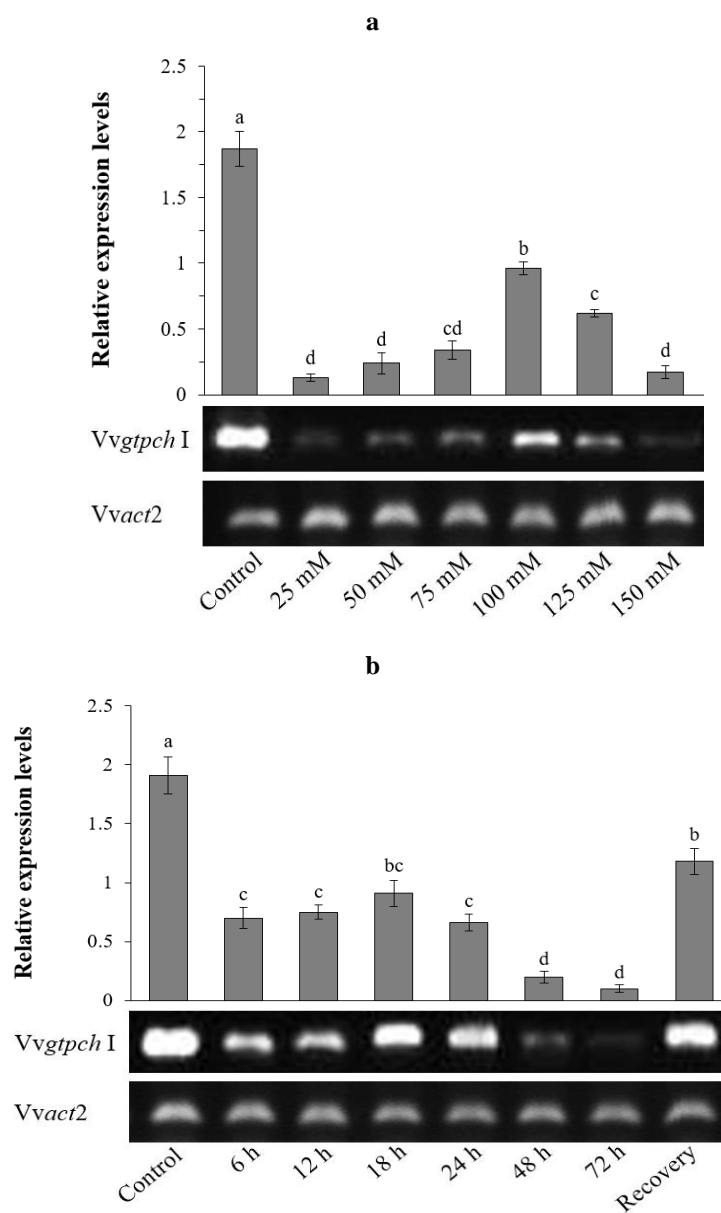
Figure 2. Expression of the Vv*gtpch* I gene under abiotic stresses by the semi-quantitative RT-PCR. (a) Expression of Vv*gtpch* I gene under alkali stress. The alkali stress was applied by exposing the cuttings to different concentrations NaHCO$_3$, including 25, 50, 75, 100, 125, and 150 mM. (b) Expression analysis of Vv*gtpch* I gene upon low temperature and after recovery. The cold stress was applied by exposing the cuttings to a temperature of 4 °C for 6, 12, 18, 24, 48, and 72 h in a chamber growth. The experiments were carried out in three independent replicates. Bars with the same lowercase letter are significantly different ($p \leq 0.05$). Each histogram represents the mean ± SD obtained from three independent PCR reactions.

Arabidopsis under cold stress (Changsong and Diqiu 2010) and *Arabidopsis* seeds under drought and heat stresses (Navarrete *et al.* 2012).

Expression of Vv*gtpch* I gene under oxidative stresses

The Vv*gtpch* I showed a strong increase and a moderate increase in the transcript amount

with Cu^{2+} and H_2O_2, respectively. Whereas, its transcript level was relatively down-regulated by heavy metals and hormonal treatments, and almost disappeared by diamide (Figure 3). In contrast, by the semi-quantitative RT-PCR method, we already revealed that the transcripts amount of Vv*gtpch* I gene raised under H_2O_2, $CuSO_4$, $CdCl_3$, and $CoCl_2$ treatments, while its expression decreased under $AlCl_3$, ABA, and SA (Eslami-Bojnourdi *et al*. 2017). The H_2O_2 regulates *gtpch* I activity in a concentration-dependent manner. Up to 300 mmol L^{-1} activates *gtpch* I function,

but higher concentrations inhibit the enzyme activity (Chavan *et al*. 2009). This observation was similar to many other enzymes, where the low H_2O_2 concentrations are beneficial while higher concentrations are inhibitory (Schallreuter and Elwary 2007). Chavan *et al*. (2009) showed that 100 mM L^{-1} H_2O_2 increased the enzyme activity, whereas concentrations >300 mM L^{-1} decreased it. They also reported that *gtpch* I activity levels remained high even after the reduction of H_2O_2.
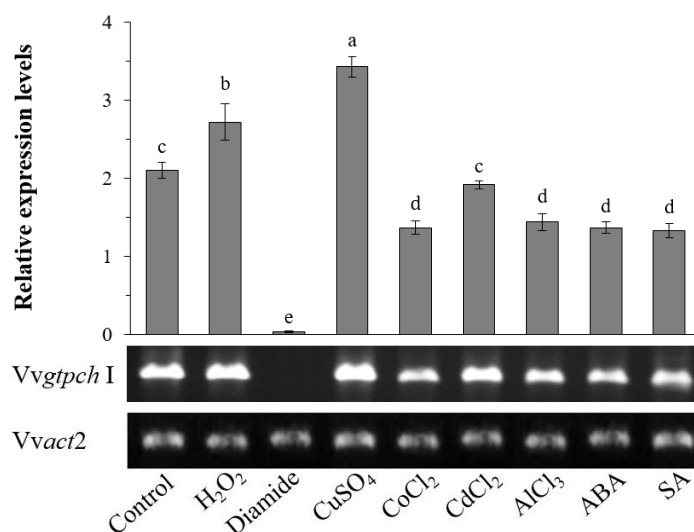

Figure 3. Expression of the Vv*gtpch* I gene under oxidative stressors by the semi-quantitative RT-PCR. Leaf slices were incubated in the presence of H_2O_2 (10 mM), diamide (1 mM), $CuSO_4$ (100 µM), $CoCl_2$ (100 µM), $CdCl_2$ (100 µM), $AlCl_3$ (100 µM), ABA (100 µM), and SA (100 µM) as mediators of the oxidative stress for 4 h before total RNA extraction. The experiments were carried out in three independent replicates. Bars with the same lowercase letter are significantly different ($p \leq 0.05$). Each histogram represents the mean ± SD obtained from three independent PCR reactions.

Conclusion

The Vv*gtpch* I gene was found to be differentially excited under abiotic stress conditions probably due to the presence of diverse putative regulatory elements in its promoter region. The transcript level of

Vv*gtpch* I gene decreased under abiotic stresses including alkali and cold. Moreover, under oxidative stresses, the expression level of this gene enhanced under H_2O_2 and $CuSO_4$, while it decreased under other heavy metals and plant growth regulators (ABA and SA).

The analysis of the promoter region of grape *gtpch* I demonstrated that there are several potential *cis*-acting elements respondent to environmental signals, including heat, heavy metals, light, and plant hormones (auxin and salicylic acid) (data not published). Collectively, due to the presence of different potential *cis*-acting elements in its promoter region, it was observed that the Vv*gtpch* I gene was down-regulated under abiotic stress conditions but it was up-regulated in the presence of H_2O_2. Thus, it is concluded that the Vv*gtpch* I gene does not play a role in increasing the tolerance of plants to abiotic stress conditions and probably has a role in the regulation of cell redox.

Conflict of interest

There is no potential conflict of interest by the authors.

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پاسخ متفاوت ژن *GTP* سیکلوهیدرولاز I تحت تنش‌های غیرزنده در انگور

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چکیده

فولات یک نیاز ضروری برای حفظ فعالیت طبیعی سلول به شمار می‌رود. برخلاف انسان، بسیاری از موجودات زنده مانند گیاهان توانایی ساخت این کوآنزیم را دارند. آنزیم GTP سیکلوهیدرولاز I (gtpch I, EC 3.5.4.16) اولین مرحله بیوسنتز تتراهیدروفولات (FH_4) در گیاهان را از طریق تبدیل GTP به دی‌هیدرونئوپترین تری‌فسفات و فرمیک اسید کاتالیز می‌کند. در این پژوهش، الگوی بیان ژن Vv*gtpch* I در بافت‌های مختلف انگور با روش RT-PCR نیمه‌کمی مورد بررسی قرار گرفت. نتایج نشان داد که ژن Vv*gtpch* I در تمام بافت‌های مورد مطالعه بیان می‌شود. بالاترین سطح بیان در بافت‌های حبه و برگ مشاهده شد. در حالی که بافت خوشه کمترین میزان سطح رونوشت را نشان داد. پاسخ ژن Vv*gtpch* I به تنش‌های غیرزنده مانند تنش‌های قلیایی و سرما با استفاده از روش RT-PCR نیمه‌کمی نیز مورد بررسی قرار گرفت. تحت تنش قلیایی، سطح رونوشت ژن Vv*gtpch* I به طور چشمگیری کاهش یافت. مشابه با تنش قلیایی، میزان بیان ژن Vv*gtpch* I نیز تحت تنش سرما با کاهش مواجه شد. به منظور بررسی بیان ژن Vv*gtpch* I تحت تنش اکسیداتیو، تیمارهای مختلفی شامل القاءکننده‌های شیمیایی، فلزات سنگین و تنظیم‌کننده‌های رشد گیاهی جهت القاء تولید گونه‌های فعال اکسیژن مورد استفاده قرار گرفتند. سطح رونوشت ژن Vv*gtpch* I تحت تیمار با CU^{2+} و H_2O_2 به ترتیب با افزایش شدید و نسبتاً شدید روبرو شد. با وجود این، سطح رونوشت ژن Vv*gtpch* I تحت تیمار با فلزات سنگین و هورمون‌های گیاهی نسبتاً کاهش یافت و تحت تیمار با Diamide با کاهش بسیار شدید مواجه شد.

واژه‌های کلیدی: بیان ژن، تنش اکسیداتیو، تنش غیرزنده، فولات، گونه‌های فعال اکسیژن