Journal of Genetic Engineering and Biotechnology (2017) 15, 257–261

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and
National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

Salicylic acid affects the expression of *VvCBF4* gene in grapes subjected to low temperature

Mohammad Ali Aazami, Nasser Mahna *

Department of Horticultural Sciences, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Received 25 February 2016; revised 30 December 2016; accepted 4 January 2017
Available online 23 January 2017

KEYWORDS

qRT-PCR;
VvCBF4;
Salicylic acid;
Vitis viniferea;
Cold stress

Abstract The present study investigates the effects of exogenous salicylic acid (SA) on the expression of *Vitis vinifera C-repeat binding factor 4* (*VvCBF4*) gene under low-temperature conditions in an Iranian *Vitis viniferea* L. 'Sultanina'. The experiment was conducted as a factorial experiment based on a completely randomized design with four replications. 100 μmol/L SA (0, 1, 6 and 12 h before applying cold stress) in temperatures of 1 ± 0.5 °C (for 1, 3, 6 and 12 h) and 22 °C (as control) were applied. The highest expression was observed in plants treated 6 h before sampling. By increasing the duration of low temperature, the expression of *VvCBF4* increased. Increasing the duration of cold stress to 6 h in 1 °C increased the expression of *VvCBF4* to 24.3 fold. Exogenous application of SA and cold stress treatments increased the expression of *VvCBF4*. In conclusion, exogenous application of SA in cold stress, increased the expression of *VvCBF4* depending on treating time before cold stress. The highest *VvCBF4* expression was observed in plants treated 6 h before sampling and increasing the time decreased the expression. By increasing the expression of *VvCBF4* the tolerance of plant to cold stress increased.

1. Introduction

Low temperature is one of the most important environmental stresses that limits the productivity and distribution of plants [22]. Various plant species can increase their freezing tolerance in response to low non-freezing temperatures; this phenomenon is defined as cold acclimation. Some molecular and physiological changes are involved in cold acclimation [27].

Although, the molecular basis of this acquired chilling acclimation is poorly understood, but the effect of some transcription factors involved in response to low temperature is well established [17,23]. *C-Repeat Binding Factors* (*CBF*s) are transcription factors that have a vital role in gene regulation during cold acclimation in plant species [3,2,7]. Constitutive expression of either *CBF1* or *CBF3* transcriptional activators in transgenic Arabidopsis induced the expression of cold-regulated genes and also enhanced the freezing tolerance in non-acclimated plants [1,8,13]. In *CBF3*-expressing plants the proline and total soluble sugars had raised, also in cold-acclimated plants with overexpressed *CBF3*, freezing tolerance have been increased [8]. Furthermore, the ectopic expression of *CBF*s from other plant species can increase the freezing tolerance of transgenic Arabidopsis [26].

* Corresponding author.
E-mail address: n.mahna@gmail.com (N. Mahna).

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2017.01.005
1687-157X

Salicylic acid (SA) is an endogenous simple phenolic acid with hormonal function and omnipresent distribution among plants [24]. SA is involved in the regulation of major variety of metabolic and physiological processes in plants. Numerous studies have reported the valuable effects of SA treatments on cold tolerance of plants species such as tomato [4,19], wheat [20], banana [11], maize [18] and so on.

Grape is one of the most cultivated fruit crops that its importance is well known. Nevertheless, the cold stress always affects the growth, development, and productivity of this plant and limits its geographical distribution [6]. All in all, the present study was designed to investigate the effects of exogenous SA on expression of *VvCBF4* gene under low temperature conditions in an Iranian grape (Sultanina cultivar).

2. Materials and methods

2.1. Plant materials and study design

The experiment was conducted in a controlled-environment on two year old greenhouse-grown plants (*Vitis viniferea* L. 'Sultanina') under day and night temperature of 28–25 °C and 20–18 °C, respectively and maintained under a 16:8 light/dark cycle. The application of SA was through spraying of 100 µmol/L SA (0, 1, 6 and 12 h before applying cold stress) in temperatures of 1 ± 0.5 °C (for 1, 3, 6 and 12 h) and 22 °C (as control) in a factorial experiment based on completely randomized design in four replications.

2.2. RNA extraction and DNA synthesis

Total RNAs were extracted and purified from the leaves of grapes following the method described by Tattersall et al. [21]. Only the extractions having an A_{260}/A_{280} ratio of 1.8–2.0 and an A_{260}/A_{230} ratio > 2.0 were applied 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 1) was prepared in a microtube on ice and was made up to 20 µl using RNase-free water.

2.3. Primer design and RT-qPCR analysis

The RNA sequences of *VvCBF4* gene were taken from NCBI (www.ncbi.nlm.nih.gov) and its forward and reverse primers was designed by Oligo 7 (Table 2).

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 3) was made up to 20 µl 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 (Fig. 1). 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 [15]. 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 1 Required amounts of reactives for cDNA synthesis.

Reactive	Volume
Vivantis RT Enzyme Mix I	0.5 µl
Buffer RT Enzyme	2 µl
Oligo dT Primer (50 µ M)	0.5 µl
Random 6 mers (100 µ M)	0.5 µl
dNTP	1 µl
DDW	11.5 µl
Total RNA (500 ng)	5 µl
Total	20 µl

Table 2 Used primers for RT-PCR reaction.

Primer name	Sequence
VvCBF4 F	5-ACCCTCACCCGCTCGTATG-3
VvCBF4 R	5-CCGCGTCTCCCGAAACTT-3

Table 3 The composition of reaction mixture for RT-PCR reaction.

Volume	Reactive
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

3. Results and discussion

Low temperature is one of the most important environmental stresses that hampers the manifestation of full genetic potential in plants. In recent studies, transcriptome analyses of cold response have shown that some transcription factors such as *CBF*s are involved in response to low temperature [26]. Also the effects of SA in ameliorating environmental stresses have been numerously reported [10].

Putting this all together, in the present investigation, we have decided to address the effects of exogenous SA on the expression of the *VvCBF4* gene under low temperature conditions in *Vitis vinifera* L. 'Sultanina'.

The exogenous SA, activated the *VvCBF4* gene in control plants (22 °C). The highest expression was observed in the plants treated 6 h before sampling. By increasing the time of SA treatment before sampling, a significant decrease in the expression of *VvCBF4* was observed (Fig. 2). Cold stress (1 °C) for 1 h along with SA also increased the expression of *VvCBF4*, however, had some decrease in comparison with control plants. The highest *VvCBF4* expression belonged to 1 h treating in 1 °C and application of SA 6 h before sampling (Fig. 3). It has been demonstrated that pretreatment with 0.1 mM SA would induce the chilling tolerance in potato plants [16]. Also in banana seedlings, 0.5 mM SA has been reported to induce the chilling tolerance both when sprayed

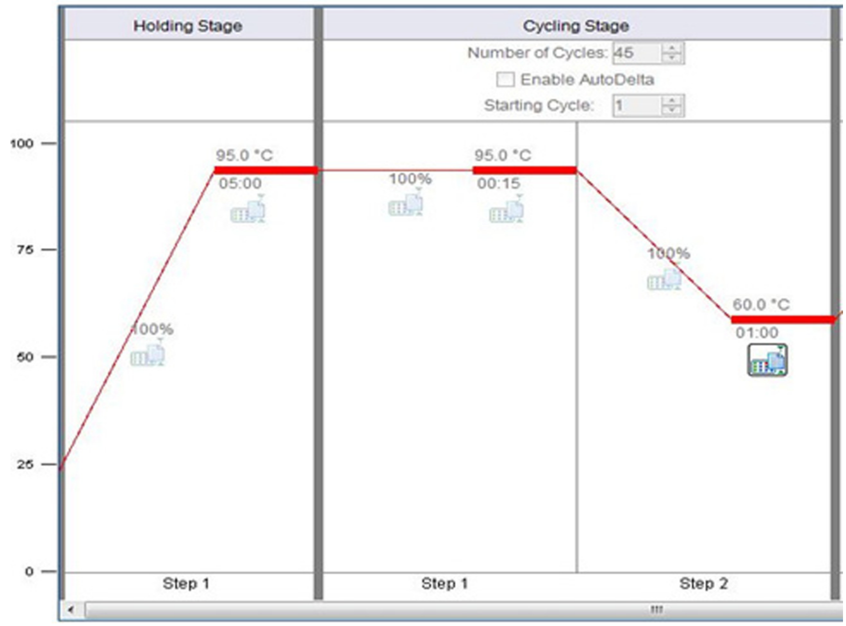

Figure 1 The program of real time PCR device in different steps of the PCR reaction.

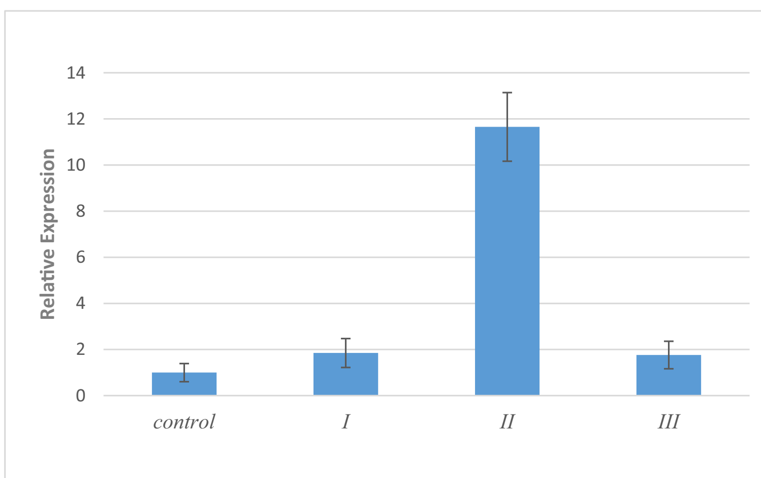

Figure 2 The expression pattern of *VvCBF4* in response to SA at 22 °C I, II and III, are plants treated with SA at 1, 6, and 12 hours before cold stress, respectively.

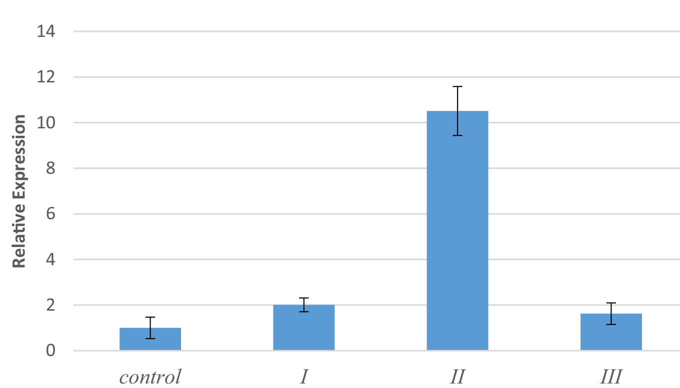

Figure 3 The expression pattern of *VvCBF4* in response to SA at 1 °C I, II and III, are plants treated with SA at 1, 6, and 12 hours before 1 °C cold stress for 1 h.

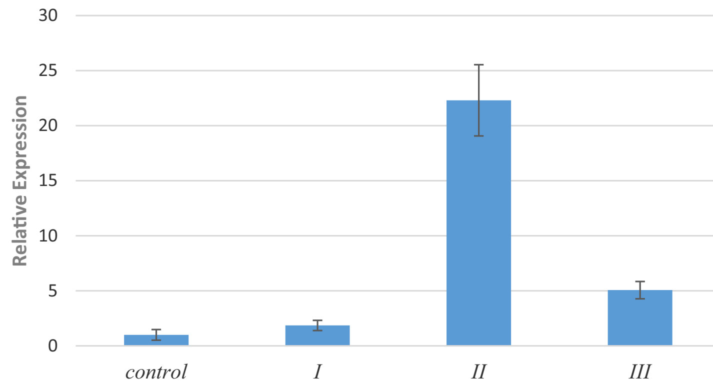

Figure 4 The expression pattern of *VvCBF4* in response to SA at 1 °C I, II and III, are plants treated with SA at 1, 6, and 12 hours before 1 °C cold stress for 3 h.

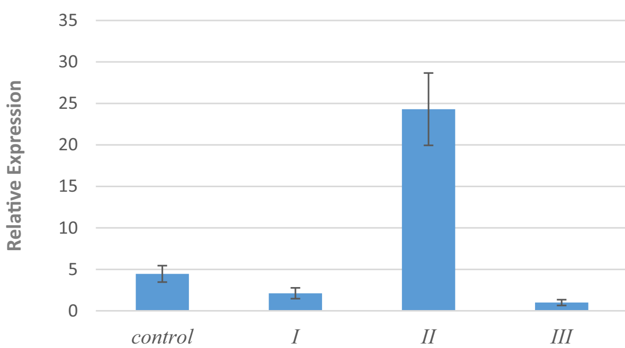

Figure 5 The expression pattern of *VvCBF4* in response to SA at 1 °C I, II and III, are plants treated with SA at 1, 6, and 12 hours before 1 °C cold stress for 6 h.

onto the leaves and also when applied in irrigation to the roots [12].

By increasing the duration of low temperature, the expression of *VvCBF4* increased, so that in 3 h treating in 1 °C and application of SA 6 h before sampling, the highest expression (22.3 fold) was observed (Fig. 4). Increasing the duration of cold stress to 6 h in 1 °C increased the expression of *VvCBF4* to 24.3 fold (Fig. 5). Meanwhile increasing the duration of cold stress to 12 h in 1 °C in comparison with 6 h in 1 °C decreased the expression of *VvCBF4* (Fig. 6).

Comparing the low temperature treated to control indicated that by increasing the duration of cold stress the expression of *VvCBF4* would increase. The highest expression of *VvCBF4* (13.9) was observed in 6 h in 1 °C which increasing the duration of cold stress to 12 h have decreased (9.9) the expression of *VvCBF4* (Fig. 7). It has been reported that cold, exogenous abscisic acid (ABA), drought, and salinity conditions would induce the expression of *VaCBF4*. Overexpressing the *VaCBF4* in transgenic *Arabidopsis* increased the tolerance to cold, salinity, and drought compared to wild-type controls [14].

The overexpression of *CBF4* under CaMV 35S promoter caused the expression of cold and drought induced genes in non-stress conditions in Arabidopsis. Also the transgenic plants were more tolerant to drought and freezing [9]. Our results confirm the expression of *VvCBF4* and its importance in cold stress situation. Our results clearly indicate that *VvCBF4* is involved in the response to cold stress, so that one hour cold stress induced the *VvCBF4* expression, in 6 h cold stress the *VvCBF4* expression reached to the highest level and more than 6 h cold stress decreased the *VvCBF4* expression. In a study *Vitis CBF* genes have been reported to accumulate relatively quickly after cold treatment that is keeping with our finding, whereas their finding about its constancy

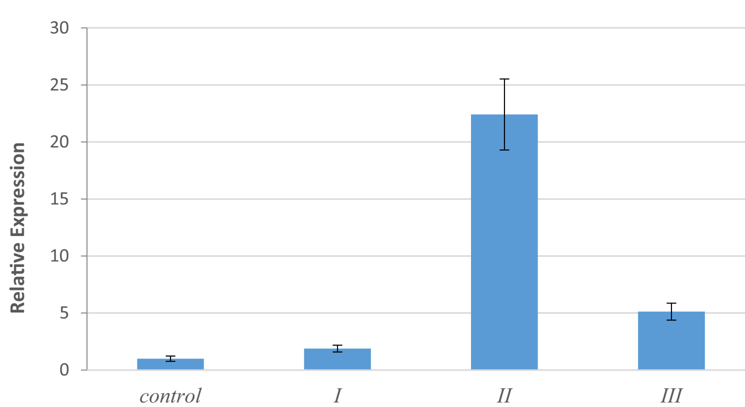

Figure 6 The expression pattern of *VvCBF4* in response to SA at 1 °C I, II and III, are plants treated with SA at 1, 6, and 12 hours before 1 °C cold stress for 12 h.

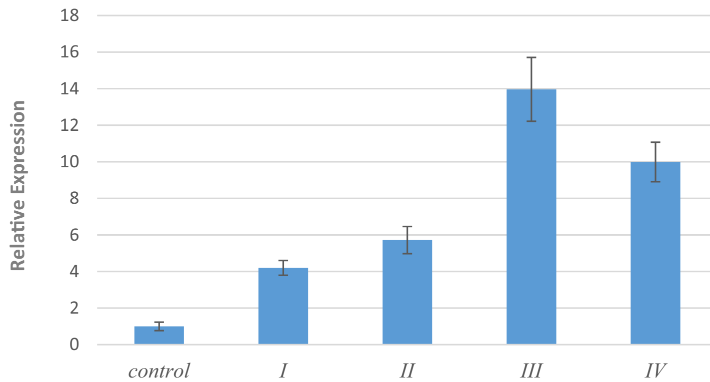

Figure 7 The expression pattern of *VvCBF4* in response to SA at 1 °C I, II III and IV, are the duration of cold treatment in 1 °C.

for a long time period, more than a day is in contrary to our results and our finding indicates that long duration treatment than 6 h would decreases the expression of *VvCBF4* [25]. Low-temperature stress would affect the membrane fluidity and causes rapid increase in cytosolic calcium, which acts as a secondary messenger in plants. Members of the *calmodulin binding transcription activator* (*CAMTA*) family have a distinct role in cold acclimation and integrates the low-temperature calcium and calmodulin signaling with cold-regulated genes such as *CBF*s [5].

Exogenous SA has potentially reduced the damaging effects of cold stress in several crops [20,18,12], and in our study it seems that SA could increase the expression of transcription factors such as *VvCBF4* and induced the tolerance to cold stress in 'Sultanina' cultivar.

In conclusion, exogenous application of SA in cold stress treatments, would increase the expression of *VvCBF4*. While, suitable SA pretreatment depends on the treating time before cold stress. The highest *VvCBF4* expression observed in plants treated 6 h before sampling and by increasing time the expression decreased. By increasing the expression of *VvCBF4* the tolerance of plant to cold stress increased.

References

[1] N.N. Artus, M. Uemura, P.L. Steponkus, S.J. Gilmour, C. Lin, M.F. Thomashow, Proc. Natl. Acad. Sci. 93 (23) (1996) 13404–13409.
[2] V. Chinnusamy, J. Zhu, J.K. Zhu, Physiol. Plant. 126 (1) (2006) 52–61.
[3] V. Chinnusamy, J. Zhu, J.-K. Zhu, Trends Plant Sci. 12 (10) (2007) 444–451.
[4] C.K. Ding, C.Y. Wang, K.C. Gross, D.L. Smith, Planta 214 (6) (2002) 895–901.
[5] C.J. Doherty, H.A. Van Buskirk, S.J. Myers, M.F. Thomashow, Plant Cell 21 (3) (2009) 972–984.
[6] C. Dong, M. Zhang, Z. Yu, J. Ren, Y. Qin, B. Wang, et al, Agric. Sci. 4 (05) (2013) 224.
[7] S. Fowler, M.F. Thomashow, Plant Cell 14 (8) (2002) 1675–1690.
[8] S.J. Gilmour, A.M. Sebolt, M.P. Salazar, J.D. Everard, M.F. Thomashow, Plant Physiol. 124 (4) (2000) 1854–1865.
[9] V. Haake, D. Cook, J. Riechmann, O. Pineda, M.F. Thomashow, J.Z. Zhang, Plant Physiol. 130 (2) (2002) 639–648.
[10] E. Horváth, G. Szalai, T. Janda, J. Plant Growth Regul. 26 (3) (2007) 290–300.
[11] G. Kang, Z. Wang, G. Sun, Acta Botanica Sinica 45 (5) (2003) 567–573.
[12] G. Kang, C. Wang, G. Sun, Z. Wang, Environ. Exp. Bot. 50 (1) (2003) 9–15.
[13] M. Kasuga, Q. Liu, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, Nat. Biotechnol. 17 (3) (1999) 287–291.
[14] J. Li, N. Wang, H. Xin, S. Li, Plant Mol. Biol. Rep. 31 (6) (2013) 1518–1528.
[15] K.J. Livak, T.D. Schmittgen, Methods 25 (4) (2001) 402–408.
[16] M.E. Mora-Herrera, H. López-Delgado, A. Castillo-Morales, C.H. Foyer, Physiol. Plant. 125 (4) (2005) 430–440.
[17] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, Proc. Natl. Acad. Sci. 94 (3) (1997) 1035–1040.
[18] G. Szalai, I. Tari, T. Janda, A. Pestenacz, E. Páldi, Biol. Plant. 43 (4) (2000) 637–640.
[19] T. Senaratna, D. Touchell, E. Bunn, K. Dixon, Plant Growth Regul. 30 (2) (2000) 157–161.
[20] E. Taşgín, Ö. Atící, B. Nalbantoğlu, Plant Growth Regul. 41 (3) (2003) 231–236.
[21] E.A. Tattersall, A. Ergul, F. AlKayal, L. DeLuc, J.C. Cushman, G.R. Cramer, Am. J. Enol. Vitic. 56 (4) (2005) 400–406.
[22] A. Theocharis, C. Clément, E.A. Barka, Planta 235 (6) (2012) 1091–1105.
[23] M.F. Thomashow, Plant Physiol. 154 (2) (2010) 571–577.
[24] T. Tounekti, I. Hernández, S. Munné-Bosch, Springer, Salicylic acid, 2013, pp. 141–162.
[25] H. Xiao, E.A. Tattersall, M.K. Siddiqua, G.R. Cramer, A. Nassuth, Plant Cell Environ. 31 (1) (2008) 1–10.
[26] K. Yamaguchi-Shinozaki, K. Shinozaki, Annu. Rev. Plant Biol. 57 (2006) 781–803.
[27] J. Zhu, C.-H. Dong, J.-K. Zhu, Curr. Opin. Plant Biol. 10 (3) (2007) 290–295.