FEBS Letters 583 (2009) 3329–3334

FEBS Letters

journal homepage: www.FEBSLetters.org

Ethylene and cold participate in the regulation of *LeCBF1* gene expression in postharvest tomato fruits

Danying Zhao [a,b,1], Lin Shen [a,c,1], Bei Fan [d], Mengmeng Yu [a], Yang Zheng [a], Shengnan Lv [a], Jiping Sheng [a,*]

[a] *College of Food Science and Nutritional Engineering, China Agriculture University, Beijing 100083, China*
[b] *Beijing Xicheng District Disease Control and Prevention Center, Beijing 100011, China*
[c] *Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, The Chinese Academy of Sciences, Beijing 100093, China*
[d] *Institute of Agro-Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China*

ARTICLE INFO

Article history:
Received 6 July 2009
Revised 3 September 2009
Accepted 3 September 2009
Available online 18 September 2009

Edited by Ulf-Ingo Flügge

Keywords:
Tomato
Postharvest fruit
Cold tolerance
Cold storage
Lycopersicon esculentum putative transcriptional activator *CBF1*

ABSTRACT

C-Repeat/dehydration-responsive element binding factor (*CBF*) is a transcription factor regulating cold response in plants, of which little is known in fruits. We showed a double-peak expression pattern of *Lycopersicon esculentum* putative transcriptional activator *CBF1* (*LeCBF1*) in mature green fruit. The peaks appeared at 2 and 16 h after subjection to cold storage (2 °C). The second peak was coincident with, and thus caused by a peak in endogenous ethylene production. We showed that *LeCBF1* expression was regulated by exogenous ethylene and 1-methylcyclopropene, and was not expressed without cold induction. *LeCBF1* expression was different in the five maturation stages of fruits, but expression peaked at 2 h at all stages.
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1. Introduction

Plants have evolved various protective mechanisms to cope with cold stress. However, some species from tropical regions, such as tomato, are unable to tolerate freezing and suffer chilling injury when exposed to a cold (0–12 °C) environment. Transcription factors play an important role in the cold resistant mechanisms, regulating downstream cold-responsive genes to trigger resistant processes to cold [1,2]. *Arabidopsis CBF* (C-repeat/dehydration-responsive element binding factor, *DREB1*) transcription factors recognize the C-repeat/DRE DNA regulatory elements of *COR* (cold-regulated) genes and bind to them, thus play a prominent role in regulating the cold acclimation response [3,4]. Many studies have shown that *CBF* genes widely exist in cold tolerant- and sensitive-plants, with conserved nucleic acid sequences and functions [5,7,8]. Two aspects of this research have provided convincing evidence for the crucial function of *CBF*. Firstly, employment of array-based transcript profiling technology has allowed the identification of the cold-responsive genes in upstream and downstream of *CBF*, and has provided evidence for the existence of a functional *CBF* cold response pathway [9–11]. This suggests that *CBF* genes act as key factors in regulating the cold-resistance process. Secondly, the prominent and conserved function of *CBF* in plants is argued through transgenic approaches. Overexpression of *CBF* genes from cold-tolerant or cold-sensitive plants is sufficient to induce constitutive expression of *CBF*-target genes and enhances cold tolerance in transgenic plants, no matter which are cold-tolerant or cold-sensitive species [12–15]. Therefore, *CBF* genes are important, evolutionarily conserved, components of the cold-resistance process in diverse plant species [16,17].

There is no doubt that *CBF* is vital in plants, but how important it is in fruits, is not well known. In a previous study, we discussed the function of the *CBF* gene in postharvest fruit [6]. We detected the expression of *LeCBF1* gene, which is functional in the cold response of tomato seedlings [7] and in postharvest fruits in cold storage (2 °C). We also compared the expression level in tomato fruits from two cultivars differing in their cold tolerances. The results indicated that in fruit, the gene swiftly responded to cold and its expression level was positively correlated with cold tolerance of the cultivars. Thus the *CBF* gene has the potential to be used

Abbreviations: *CBF*, C-repeat/dehydration-responsive element binding factor; COR, cold-related; Ct, threshold constant; *LeCBF1*, *Lycopersicon esculentum* putative transcriptional activator *CBF1*; QRT-PCR, quantitative (real-time) RT-PCR; RT-PCR, reverse transcription PCR; 1-MCP, 1-methylcyclopropene

* Corresponding author. Fax: +86 10 62737620.
E-mail address: pingshen@cau.edu.cn (J. Sheng).

[1] These authors contributed to this work equally.

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doi:10.1016/j.febslet.2009.09.029

as an indicator in cultivar selection and in chilling injury inspection. However, if we hope to establish method of evaluating tomato fruits' properties that is dependent on *LeCBF1* detection, we must know its gene expression pattern to determine the correct detection time and to identify major regulatory factors that might affect the utility of the method. In the present study, we determined the *LeCBF1* expression pattern in postharvest tomato fruits from all maturity stages and analyzed regulatory factors of its gene expression. The results provided us with the basic information necessary to establish the method of tomato condition evaluation.

2. Materials and methods

2.1. Plant material and treatment

Tomato fruits (*Solanum lycopersicum* cv. Lichun), free from blemishes or disease, were harvested at the mature green stage from a greenhouse in the China Agricultural University in June 2007. All fruits were selected for uniformity of shape, color (green), and size. The calyxes of the fruits were removed. Lichun fruits were randomly divided into three replicates in different baskets and stored at (2 ± 1) °C with 80–90% RH (relativity humidity) in cold storage for up to 3 weeks. The mesocarp from the fruit equator area was cut into small pieces, frozen in liquid N_2, and stored at −80 °C for enzyme and gene assays. For ethylene and 1-MCP treatments, two groups of fruits were dipped into 0.01% aqueous solution of ethephon for 10 min and treated with 500 nL L^{-1} 1-MCP (1-methylcyclopropene) at room temperature for 12 h, respectively. 1-MCP is a competitive inhibitor of ethylene binding that can block ethylene perception in plants.

Seedlings were grown in a growth chamber for 30 days with a 14 h light period under 4400 Lux light intensity at 28 °C and a 10 h dark period at 20 °C. For the cold stress treatment, uniformly developed 7-week seedlings were transferred to 4 °C at the fourth hour during the light period. New leaves were harvested directly into liquid N_2 and store at −80 °C for later use.

2.2. RT-PCR

Total RNAs were obtained by Trizol regent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out using 2 μg total RNA treated with RNase-free DNase I (Promega, Madison, WI, USA) and AMV reverse transcriptase (Promega). The volume of each cDNA pool was adjusted to give the same exponential phase PCR signal strength according to the expression level of the tomato *Ubi3* gene (accession no. X58253) as an endogenous control [18]. The analysis of gene expression level was based on the band's intensity on an ethidium–bromide-stained gel.

2.3. Quantitative (real-time) RT-PCR

Quantitative RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) [19]. For each reaction 20 ng of cDNA were used with SYBR GREENI MasterMix (Toyobo, Osaka, Japan). All experiments were run in triplicate with different cDNAs synthesized from three biological replicates. To determine relative fold differences for each sample, the threshold constant (Ct) value was normalized to the Ct value for *Ubi3*, and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

2.4. Measurement of chilling injury index

Chilling injury (CI) of fruits was evaluated at 20 °C for 3 days after 5-, 10-, 15-, and 20-days in cold-storage. The fruits were returned to ambient temperature (20 °C) for development of CI symptoms. Symptoms were manifested as surface pitting according to the method of Ding [20]. The severity of the symptoms was assessed visually using a four-stage scale: 0 = no pitting; 1 = pitting covering <25% of the fruit surface; 2 = pitting covering <50%, but >25% of surface; 3 = pitting covering <75%, but >50% of surface, and 4 = pitting covering >75% of surface. The average extent of cold damage was expressed as a CI index, which was calculated using the following formula: CI index (%) = $\{\sum[(\text{CI level}) \times (\text{Number of fruit at the CI level})]/(\text{Total number of fruits}) \times 4\} \times 100$.

2.5. Measurement of ion leakage

Ion leakage was measured immediately after 0-, 5-, 10-, 15-, and 20-days in cold storage (2 °C). The measurement method was that of Jiang with modifications [21]. Cylinders (3 mm thick) of mesocarp tissue were excised with a 1 cm diameter stainless steel cork borer from the equator part of four fruits. Disks were put into aqueous 0.1 M mannitol and shaken at 100 cycles per min for 2 h. The conductivity of the solution (L1) was measured with a conductivity meter (DDS-11A, Shanghai Leici Instrument Inc., Shanghai, China). Solutions were boiled for 10 min and then cooled to 20 °C. The conductivity of killed tissues (L2) was measured. Ion leakage was calculated as the ratio of L1 to L2.

2.6. Measurement of malondialdehyde content

Content of malondialdehyde (MDA) was measured immediately after 0-, 5-, 10-, 15-, and 20-days of cold storage (2 °C) using the thiobarbituric acid method described by Ding with modifications [22]. Absorbance at 532 nm was recorded and corrected for non-specific absorbance at 600 nm. The amount of MDA was calculated from the extinction coefficient of 0.0155 μmol l^{-1} and expressed as μmol g^{-1}, where one unit was defined as 1 μmol MDA per g of pulp.

2.7. Measurement of proline content

The proline content was measured at the same time as the MDA measurement, using the acid ninhydrin method described by Bates with modifications [23]. Proline in tissues was extracted using 3% sulfosalicylic acid at 100 °C for 10 min with shaking. The extract was mixed with an equal volume of glacial acetic acid and acid ninhydrin reagent, and boiled for 30 min. After cooling, the reaction mix was partitioned against toluene, and the absorbance of the organic phase was recorded at 520 nm. The resulting values were compared with a standard curve constructed using known amounts of proline (Sigma, St Louis, MO, USA).

2.8. Statistical analysis and experiment replicates

A completely randomized design with three replicates per cultivar, where each basket constituted a replicate, was performed. Data were analyzed for significant differences by one-way analysis of variance (ANOVA) using the statistical software SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Significant effects were determined using LSD multiple comparison procedure at the 5% level and 1% level were considered significant and extremely significant, respectively.

3. Results

3.1. Double-peak expression pattern of LeCBF1 in mature green fruits

The *LeCBF1* expression pattern in mature green fruit was analyzed and compared with that of seedlings by QRT-PCR. As shown

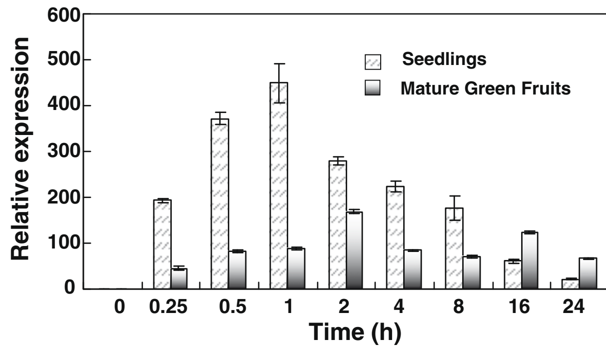

Fig. 1. Expression of *LeCBF1* in mature green tomato fruits compared with that in seedlings induced by cold stress. Representative results of QRT-PCR. All experiments were run in triplicate with different cDNAs synthesized from three biological replicates. Data represent means ± S.D., $n = 3$, normalized to the level of *LeCBF1* expression in 0 h samples.

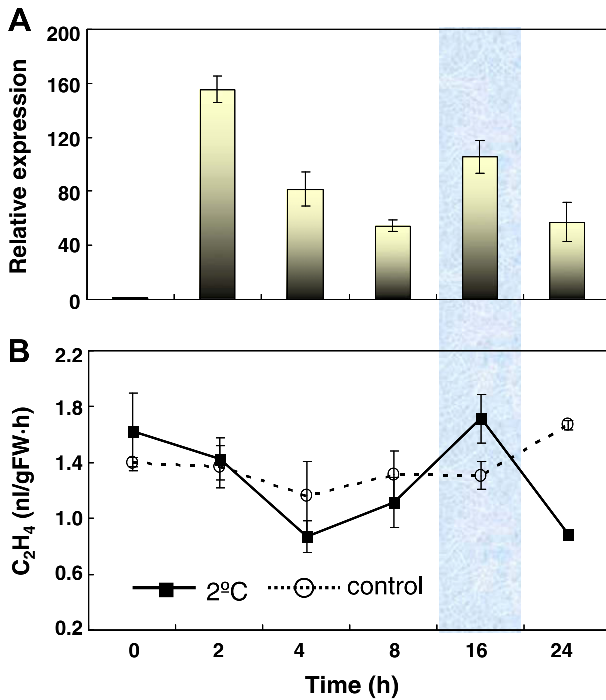

Fig. 2. *LeCBF1* expression and endogenous ethylene production in mature green tomato fruits under cold stress. (A) Relative expression level of *LeCBF1* in mature green fruits under cold stress analyzed by QRT-PCR. Blue shadow represents the time when second peak of *LeCBF1* expression and endogenous ethylene increased sharply at 4 °C. (B) Endogenous ethylene production of mature green tomato fruits with and without cold stress. Data represent means ± S.D., $n = 3$, normalized to the level of *LeCBF1* expression in 0 h samples.

in Fig. 1, the expression pattern of *LeCBF1* in mature green fruits exhibited a double-peak mode. The peaks of gene expression appeared at 2 and 16 h, respectively, within 24 h. This pattern was different from that in seedling, which showed a single-peak mode with the highest expression level after 1 h during a 24-h period of cold induction.

3.2. Ethylene and cold are regulatory factors for LeCBF1

Ethylene has an important and special role in physiological and genetic regulation in postharvest fruits, and it is one of the most significant differences between postharvest fruits and seedlings. We hypothesized that the double-peak expression pattern of *LeCBF1* in fruits might be related to endogenous ethylene. Therefore, we measured endogenous ethylene production of fruits treated with cold, and compared its pattern with *LeCBF1* expression. Fig. 2 shows that there was a swift rise of endogenous ethylene production at 16 h, coincident with the second peak of *LeCBF1* expression. This coincidence suggested that there might be a correlation between the double-peak expression pattern and endogenous ethylene production. To confirm the correlation, we treated fruits with ethephon and 1-MCP, respectively, and determined the gene expression and chilling injury of the fruits. The results showed that exogenous application of ethephon at an effective concentration could induce *LeCBF1* expression at room temperature (RT), enhance the expression under cold stress, and decrease the chilling injury of fruits (Fig. 3). Treatment with 1-MCP, a competitive inhibitor of ethylene binding that blocks ethylene perception, remarkably decreased the *LeCBF1* expression level (Fig. 3). These results indicated that ethylene was an endogenous regulatory factor of *LeCBF1*, and the absence of ethylene negatively affected *LeCBF1* expression. Ethylene was also a necessary factor for cold resistance of fruits. Exogenous ethylene treatment decreased the degree of chilling injury; the chilling injury index, ion leakage, and MDA content were all lower in fruits treated with ethephon, and the cryoprotection substance proline showed increased accumulated (Fig. 4). Using 1-MCP to block ethylene signaling increased fruit chilling injury (Fig. 4). In addition to ethylene, cold was another important regulatory factor of *LeCBF1* expression. *LeCBF1*, in common with other *CBF* genes [3–5,7–9] was not expressed at all without cold treatment (Fig. 3).

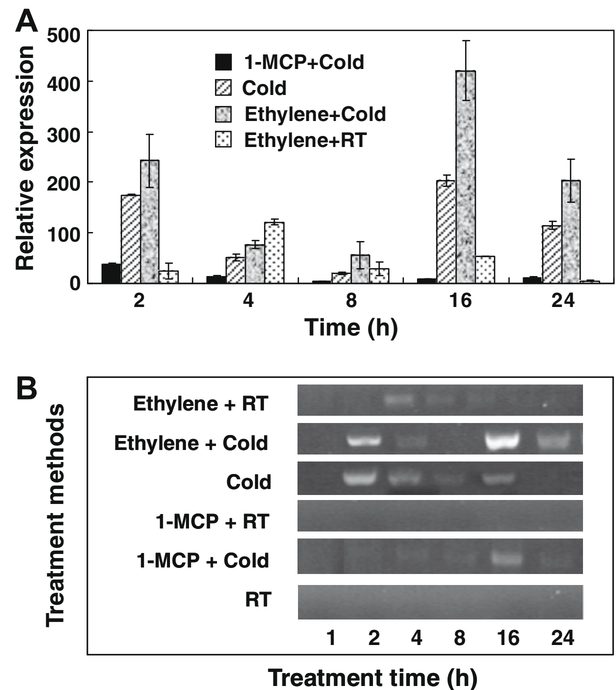

Fig. 3. Affect of ethephon and 1-MCP treatments to *LeCBF1* expression in mature green tomato fruits. (A) Relative expression level of *LeCBF1* in mature green fruits with different treatments. Data represent means ± S.D., $n = 3$, normalized to the level of *LeCBF1* expression in 0 h samples. (B) The results were from semi-quantitative RT-PCR, intensities of bands represented expression level after a 30-cycle PCR reaction. Ethylene + RT: fruits were dipped into 0.01% aqueous solution of ethephon for 10 min and stored at room temperature (RT); Ethylene + Cold: fruits were treated with ethephon as described forward and stored at 2 °C; Cold: fruits were stored at 2 °C; 1-MCP + RT: treated with 500 nl l^{-1} 1-MCP (1-methylcyclopropene) at RT for 12 h and stored at RT; 1-MCP + Cold: fruits were treated with 1-MCP as described forward and stored at 2 °C; RT: fruits without any treatments were stored at RT.

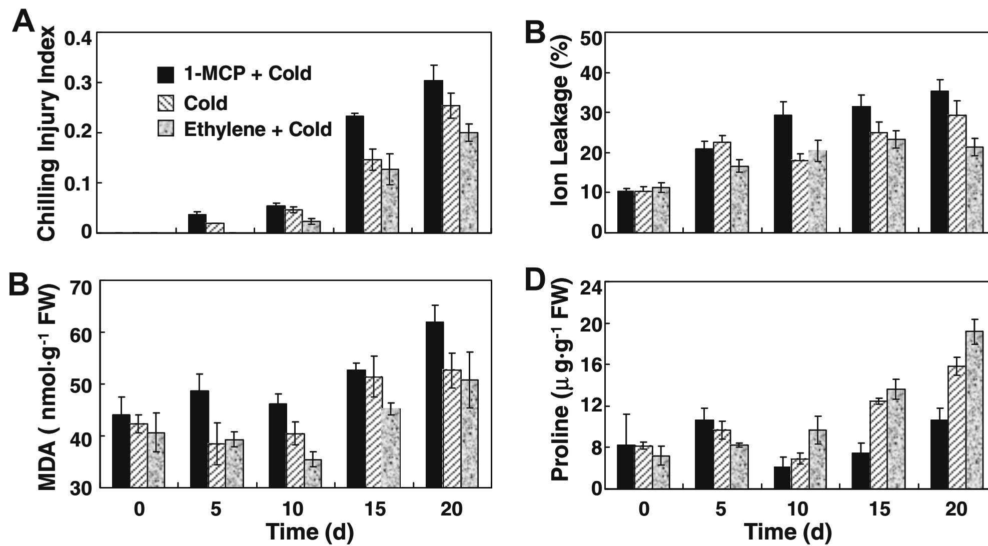

Fig. 4. Chilling injury degree of mature green fruits with treatment. 1-MCP + Cold: fruits were treated with 1-MCP as described forward and stored at 2 °C; Cold: fruits were stored at 2 °C; Ethylene + Cold: fruits were treated with ethephon as described forward and stored at 2 °C. (A) Chilling injury (CI) index; (B) Ion leakage; (C) MDA content; (D) Proline content. Data represent means ± S.D., *n* = 3.

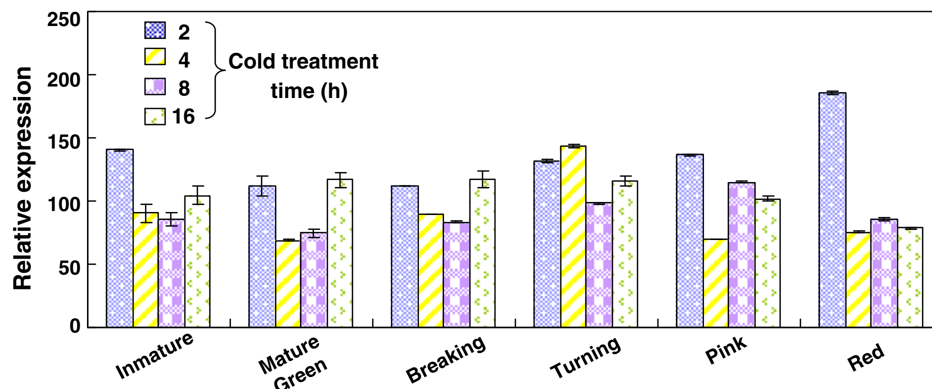

Fig. 5. Expression of *LeCBF1* in tomato fruits at different maturity stages treated under cold stress. Relative expression level of *LeCBF1* in fruits treated under 2 °C analyzed by QRT-PCR. Data represent means ± S.D., *n* = 3, normalized to the level of *LeCBF1* expression in 0 h samples.

3.3. Expression pattern of LeCBF1 in all maturity stages of tomato fruits

To obtain more information about *LeCBF1* expression in fruits, we analyzed the expression of the gene in fruits at six different maturity stages. The results showed that *LeCBF1* expression was induced by cold in fruits throughout the course of maturation (Fig. 5). However, fruits of distinct maturity stages displayed marked differences in gene expression levels. The double-peak pattern existed in fruits at most maturity stages except the turning and red stages. However, fruits at different stages did not show peaks at the same time. In fruits at immature, breaking, and mature green stages, the two expression peaks of *LeCBF1* appeared at 2 and 16 h, respectively. However, they appeared at 2 and 8 h in pink fruits. Thus, no matter when the second peak appeared, the first peak always appeared at around 2 h in all fruits stages. This indicated that, in terms of developing a method for evaluating cold-tolerant tomato cultivars, detecting *LeCBF1* expression 2 h after exposure to cold was more effective.

4. Discussion

The *CBF* gene family plays a prominent role in cold resistant process by triggering the cold response transcriptional pathway [24–26]. In a previous study, we found that *LeCBF1* had a role in the cold response of postharvest tomato fruits and discussed the possibility of using a gene detection method to select cold-tolerant tomato cultivars and for chilling injury inspection. Thus the present study attempted to solve two basic problems for the establishment of the method; when to detect the gene and what might affect the result of gene detection?

To answer the first question, we determined the *LeCBF1* expression pattern in postharvest fruits at the mature green stage. The pattern of *LeCBF1* expression was not as simple as it was in the seedlings, which showed a first-upward and last-downward trend, consistent with a study by Zhang [7]. In fruit, the expression of *LeCBF1* exhibited a double-peak mode (Fig. 1), in which the first peak appeared at 2 h after cold induction, then declined until another sharp increase appeared at 16 h. The double-peak expression mode of *CBF* genes was also found in Arabidopsis plants by McKhann

[27]; however, the reason why there were multiple peaks of *CBF* expression was not discussed. Although there was also a double-peak expression pattern in postharvest tomato fruits, the physiological or genetic regulation mechanisms regulating or affecting this pattern are probably different. The specific release of endogenous ethylene induced by cold stress in postharvest fruit might explain this gene expression pattern.

In our study, we found that the double-peak expression pattern of *LeCBF1* in fruits was related to endogenous ethylene. Endogenous ethylene production increased at 16 h after cold treatment, coincident with the second peak of *LeCBF1* expression (Fig. 2). This coincidence revealed the possibility that endogenous ethylene might be the cause of the double-peak pattern of *LeCBF1* expression. Exogenous application of ethylene at the appropriate concentration could induce *LeCBF1* expression without cold treatment and enhanced its expression under cold stress (Fig. 3), suggesting that the hypothesis was correct. However, a problem still existed with such explanation, because the production of endogenous ethylene could increase to a high level in fruits without cold treatment (Fig. 3). It could be argued that increased endogenous ethylene was not a cause of the second peak of *LeCBF1* expression because high level of endogenous ethylene could not induce the gene without cold stress. Further study showed that there was no *LeCBF1* expression in fruit without cold stress and a greatly weakened expression in fruits treated with 1-MCP, which blocked ethylene perception in fruits, under cold stress (Fig. 3). These phenomena illustrated that cold and endogenous ethylene were both necessary conditions for normal expression of *LeCBF1*, and could also explain why high levels of endogenous ethylene had different effects on *LeCBF1* expression with and without cold induction. Other studies provide data supporting our hypothesis: (1) numerous studies have shown the a cold signal is a prerequisite for *CBF* genes expression [3–10,12–15]; (2) ethylene is important in the cold response process, because increased ethylene synthesis enhances the cold response of some postharvest fruits [28–30], and switching off ethylene signal transduction greatly decreases the cold response of tomato seedlings [31]. These examples showed that ethylene is necessary in the normal response to cold stress in plants. Our study also supported this because 1-MCP treatment obviously increased chilling injury of fruits (Fig. 4). (3) Gene profiling studies showed that *CBF* was related to the ethylene signaling pathway, because *CBF* shared some similar downstream genes with the ethylene signaling pathway, i.e. *ATERF4*, *ATERF5*, and *ACO2* [9,32,33]. *CBF* genes play a key role in the cold-resistance process [34,35], and a cross-talk exists between the ethylene pathway and the cold response pathway regulated by *CBF* through genes shared by the two pathways; therefore, it is reasonable to believe that ethylene is related to *CBF* in the cold response pathway. Endogenous ethylene is an important factor affecting *LeCBF1* expression, and it might provide an explanation for the double-peak expression pattern of *LeCBF1* in tomato fruit. Further studies are required to obtain more convincing proof and a detailed explanation.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant Nos. 30671471, 30571296 and 30972065), ACIAR Project (No. PHT/1999/081) and the Commonweal Industry Scientific Researcher Project from Ministry of Agriculture (No. 200803033). We thank Dr. Robert Holmes from DPI Victoria Australia for revision of this manuscript.

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