

Article

Carbon dioxide pretreatment on tomatoes before cold storage synergistically delays ripening through transcriptional change of ethylene-related genes and respiration-related metabolisms

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Abstract: The effect of CO₂ pre-treatments on tomato quality prior to cold storage was investigated using physiochemical and transcriptome changes. Three hours CO₂ treated fruits were firmer than untreated fruits and had a good appearance even after being transferred from 4°C storage to 20°C for 8 d. CO₂ pretreatment with cold storage showed a synergistic effect on delayed ripening through reduced respiration; these tomatoes exhibited a lower lycopene content than untreated fruit under cold storage. Tomatoes treated with 30% CO₂ had fewer pits than untreated fruits subjected to chilling temperatures, even after being transferred to 20°C for 8 d. Functional enrichment analyses from transcriptome and metabolome commonly showed that CO₂-responsive genes or metabolites were involved in the sucrose and starch and biosynthesis of secondary metabolisms. The most frequently detected domain, ethylene-responsive factor domain and reduced glycolysis provide insights into the mechanism that CO₂ regulates tomato quality.

Keywords: Carbon dioxide; Chilling injury; Ethylene response factor; Ripening; Tomato

1. Introduction

Tomato (*Solanum lycopersicum* Mill.) is an important crop, both nutritionally and commercially, as it serves as a good source of fiber, vitamins, beta-carotene, and lycopene. However, the tomato has a relatively short postharvest shelf life owing to its rapid ripening rate and sensitivity to cold storage conditions, which limit its transportability and marketability. During storage and transportation, ripening progresses with a color change from green to red, as well as softening and compositional changes in chemicals related to flavor and aroma, such as organic acids, sugars, and volatiles. Cold storage can maintain vegetable freshness for long periods by suppressing respiration, but it also induces a physiological disorder known as chilling injury (CI), which occurs when fruits are held at a critical temperature for too long [1]. CI in tomatoes is typified by pitting, the development of sunken areas on the fruit (blemishes), and increased susceptibility to *Alternaria* spp.-induced rot and decay [2]. These CI symptoms appear when the fruits are subjected to ripening temperatures (20–22°C) after cold storage (2–6°C) for more than 2 weeks [3]. Hence, CI symptoms usually become pronounced under market shelf conditions following cold storage, thus reducing consumer desirability [4]. However, when exporting tomatoes overseas, a long-term cold-chain transporting system is required; therefore, CI becomes a serious hurdle. Other causes of postharvest losses in tomatoes include softening, cracking, black mold rot, and gray mold rot. Thus, practical and feasible techniques to extend the shelf life of tomatoes by reducing postharvest losses and CI are required.

The benefits of exposure to high levels of carbon dioxide (CO₂) as pre- and postharvest treatments have been investigated in several commodities [5-7]. Treatment comprising exposure to high levels of CO₂ effectively controlled postharvest diseases and enhanced firmness in strawberry. This resulted in a longer shelf life without altering the color, solid soluble content (SSC), titratable acidity, or pH of the fruit [8-10]. Moreover, it decreased astringency in persimmon [5,11] as well as inhibited browning and improved the quality of fresh-cut burdock owing to a reduction in respiration rate and polyphenol oxidase (PPO) [7]. Cell wall-degrading enzyme activity was altered upon exposure to high concentrations of CO₂ in postharvest “Mihong” peach, and the delayed softening resulted in a reduction of its decay rate after 4 d of storage at 23°C [12]. Furthermore, recent studies on the postharvest management of tomatoes have shown that high levels of CO₂ reduce the decay rate of cherry tomatoes [13].

CO₂ treatment improves tolerance to prolonged cold storage. Ezz et al. [14]. suggested that treatment with high levels of CO₂ reduced chilling-induced peel pitting in grapefruit by controlling proline metabolism. Additionally, pre-storage CO₂ treatment at 10–40% was found to reduce CI symptoms in citrus fruit [15] and CO₂ treatment before storage at 2°C effectively reduced chilling-induced physiological changes in zucchini [16]. Nevertheless, at the molecular level, little is known about the response of fruits to CO₂ treatment during postharvest cold storage.

Therefore, in this study, we aimed to develop a practical postharvest technique for extending the shelf life of tomatoes. To do this, we investigated the effects of short-term exposure to CO₂ on tomato fruit quality and found that CO₂ treatment delayed ripening and reduced CI symptoms. Furthermore, to improve our understanding of the molecular mechanisms involved in the beneficial effects of CO₂ treatment on tomato quality, a comparative transcriptomic analysis and metabolite analysis between CO₂-treated and untreated plants was carried out. The results obtained improve our understanding of the manner in which tomato responds to CO₂ treatment and how tomato fruit quality can be maintained during postharvest storage.

2. Materials and Methods

2.1. Plant materials and treatments

“Defunis” tomato fruits were harvested between the mature-green and breaker stage during summer at Jungyeum, South Korea. Upon arrival at the laboratory, the fruits were immediately treated with 30% or 60% CO₂ (mixed with ambient air) or left untreated inside a commercial cardboard box covered with plastic film for 3 h in a closed chamber. The chamber was subsequently flushed with air to remove the CO₂. Thirty boxes containing 30 fruits each were used for each treatment. The CO₂ concentration in the closed chamber was verified using a portable headspace analyzer (Dansensor, Ringsted, Denmark). Control samples were flushed with only ambient air, and the damaged fruits were discarded. The fruits were stored in a commercial cardboard box covered with plastic film at 4°C (cold storage) for 14 d and/or transferred to 20°C (shelf-life conditions) for 8 d. Relative humidity was maintained at 90 ± 5% during the storage period.

2.2. Gas chromatography analysis

Respiration and ethylene production were analyzed using a gas chromatograph (Bruker 450-GC; Bruker Corp., Billerica, MA, USA). One milliliter of gas was sampled using a syringe from a 2-L container with four fruits from each treatment that had been sealed for 2 h. The injection and column temperatures were 110°C and 70°C, respectively. The thermal conductivity detector and flame ionization detector used for the CO₂ and ethylene measurements were set at 150°C and 250°C, respectively.

2.3. Fruit quality evaluation

Fifteen fruits per treatment were sampled to assess the fruit quality. Skin color was monitored using a color difference meter (Minolta CR-400; Konica Minolta, Osaka, Japan)

and reported based on Hunter's scale: redness (a^*). Firmness was analyzed using a texture analyzer (TA Plus Lloyd Instruments Ltd., Fareham, Hampshire, UK) at a speed of 2 mm/s with a plunger head 5 mm in diameter. The total SSC of the samples was analyzed using a digital refractometer (PAL-1, Atago Co. Ltd., Tokyo, Japan), while titratable acidity (TA) was determined by titrating 5 mL of juice from the fruit with 0.1 N NaOH until a pH of 8.2 was reached. This procedure was performed using an auto pH titrator (Titroline Easy; SCHOTT Instruments GmbH, Mainz, Germany), and the TA was expressed in grams of citric acid per 100 g of sample juice. CI was measured as described by Park et al. [17]: 0 = no pitting, 1 = few scattered pits, 2 = pitting covering up to 5% of the fruit surface, 3 = pitting covering 5–25% of the fruit surface, and 4 = extensive pitting covering >25% of the fruit surface. Fruit decay was expressed as the percentage of fruits showing any decay symptoms. The CI index and decay rate were taken from three replicates (three boxes of 30 fruits each) per treatment per day.

2.3. Carotenoid analysis

Carotenoids were extracted from 500 mg of dried powder samples, taken from pericarp tissues, using hexane, acetone, and ethanol (2:1:1). The hexane layer was collected, and the concentrated solution was adjusted to 2 mL (v/v) with methyl tert-butyl ether and filtered for analysis. Carotenoids were quantified using an HPLC Agilent 1200 series system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Kinetex C18 100A column (100 × 4.60 mm, 2.6 μ m; Phenomenex Inc., Torrance, CA, USA). The HPLC conditions were as follows: column temperature, 40°C; detection wavelength, 454 nm; flow rate, 0.8 mL/min; and injection volume, 20 μ L. Carotenoids were analyzed via gradient elution (70–100 %) of mobile phase solvents A (water:methanol = 25:75 [v/v]) and B (ethyl acetate). Compounds were identified by comparing their elution times with those of the verified standards.

2.4. Transcriptome analysis

Tomato fruits were sampled from the untreated control, as well as from the 30% and 60% CO₂-treated groups after 0 d, 7 d, and 14 d of storage at 4°C and were transferred to 20°C for 8 d of storage. Subsequently, five fruits were pooled from each sample, and the pericarp tissue was used for RNA isolation using the cetyl trimethylammonium bromide protocol [18]. Library preparation and RNA sequencing (RNA-Seq) were performed by Macrogen in Seoul, South Korea. Processed reads were aligned to *Solanum lycopersicum* (GCF_000188115.3_SL2.50) using HISAT v2.0.5(1) [19]. After alignment, StringTie v1.3.3b[19] was used to assemble the aligned reads into transcripts and estimate their abundance. The expression level of each transcript was normalized to values of fragments per kilobase of exon per million fragments mapped (FPKM). The filtered data were log₂-transformed and subjected to quantile normalization. Differentially expressed genes (DEGs) were selected using $p \leq 0.05$, while log₂-fold change values (FC) ≥ 2 were used as thresholds. For the DEG set, gene enrichment, functional annotation, and pathway analyses were performed using the DAVID tool (<http://david.abcc.ncifcrf.gov/>) and the Kyoto Encyclopedia of Genes and Genomes database (<http://www.genome.jp/kegg/pathway.html>). To provide a functional overview of DEGs between the CO₂ treatment and non-treatment groups, DAVID analysis was performed using all the CO₂ responsive genes, revealing a total of 4,391 DEGs based on FC >2 throughout the storage periods. Conserved domains in DEGs were analysis with InterPro using DAVID tool. Hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed transcripts with FC ≥ 2 . Data analyses and visualization of DEGs were performed using R v.3.4.3 (www.r-project.org). Expression profiling of the DEGs involved in ethylene signaling and synthesis was performed using the PermutMatrix software [20].

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described by Park et al. [17] using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA). The amplification was performed using iQ™ SYBR Green Supermix (Bio-Rad) with specific primers (Table S1). qRT-PCR was performed under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 55°C or 58°C for 40 s. Relative gene expression was calculated using the $\Delta\Delta C_t$ method and normalized using the expression levels of the house-keeping genes, *actin* and *elongation factor 1 (EF1)*. qRT-PCR analysis was performed using at least three biological replicates and two technical replicates.

2.6. Water-soluble primary metabolite profiling using gas chromatography-mass spectrometry (GC-MS)

Water-soluble primary metabolites were tested using the methods described by Lisec et al. [21]. A total of 50 mg of tomato powder was weighed into 2-mL tubes, which were then vortexed for 10 min with 1.4 mL of methanol from the freezer, while using 50 μ L of 10 mg/mL ribitol as an internal standard. Subsequently, the tubes were centrifuged at $10000 \times g$ for 3 min. A total of 700 μ L of the supernatant from the centrifuged sample was transferred to 2-mL microcentrifuge tubes. The supernatant was then vortexed for 10 s with 700 μ L of H₂O. Subsequently, 10 μ L of extract solution was transferred to 1.5-mL tubes. The samples were placed in a speed vac (Vision, Bucheon, Gyeonggi-do, Korea) at 30°C for 1 d. After drying, the samples were centrifuged at $800 \times g$ for 90 min at 37°C with 50 μ L of a freshly prepared mixture of 40 mg/mL of methoxyamide in pyridine. The sample was then centrifuged at $800 \times g$ for 20 min at 50°C with 80 μ L of N-methyl-N-(trimethylsilyl)trifluoroacetamide. For water-soluble metabolite analysis using GC-MS, the GC oven was set at an initial temperature of 80°C for 2 min, and then, the oven temperature was increased by 15°C per min up to 330°C and held for 5 min. The injector and detector temperatures were set at 205°C and 250°C, respectively. An aliquot (1 μ L) of the sample was injected at a split ratio of 200:1; meanwhile, the carrier gas (helium) was maintained at a constant flow rate of 1.2 mL/min. The mass spectrometer was operated in the positive electron impact mode at an ionization energy of 70.0 eV and a scan range of 40–500 m/z [22].

2.7. Statistical analyses

Values are presented as the mean \pm standard error. Samples were subjected to analysis of variance (ANOVA), and significant differences were determined using Duncan's multiple range test. Partial least squares-discriminant analysis (PLS-DA) and pathway analysis were conducted using MetaboAnalyst (<https://www.metaboanalyst.ca/>). All analyses were conducted using SAS v.9.2 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Respiration and ethylene production

Respiration rates were higher in tomatoes treated with 30% and 60% CO₂ than in the control fruits, indicating the successful absorption of CO₂ treatment immediately following pre-storage exposure to CO₂ treatment for 3 h (Figure 1A). However, respiration declined to a level similar to that observed in the control group after storage at 4°C for 14 d. Respiration in tomatoes stored at 4°C for 7–14 d increased and reached a climacteric peak after the fruits were transferred to 20°C for 4 d but declined thereafter. The respiration rate of CO₂-treated fruits either returned to control levels or increased after transfer to ambient air temperature.

Ethylene production was higher in fruits treated with short-term CO₂ exposure than in untreated fruits before cold storage (Figure 1B, inner box). However, this difference decreased on the second day of cold storage, and no significant difference was found among the control, 30% CO₂-treated, and 60% CO₂-treated groups of fruits during cold storage. Furthermore, fruits treated with CO₂ exhibited lower ethylene production than

the untreated control fruits when they were transferred to 20°C for 4 d after cold storage (Figure 1B).

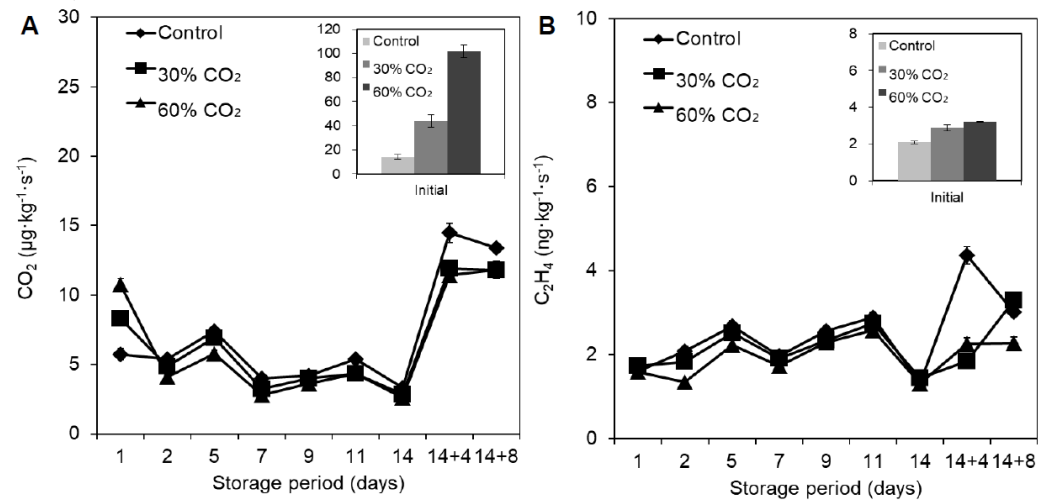


Figure 1. Respiration rate (A) and ethylene production (B) in CO_2 -treated tomatoes during storage. Samples were obtained from untreated (control) and CO_2 -treated tomatoes during storage at 4°C for 14 d and were transferred to 20°C for another 4 (14 + 4) to 8 days (14 + 8). The initial (inner box) indicates tomatoes before storage after CO_2 treatment or after no treatment. Data are shown as the mean \pm standard error (SE) of three replicates.

3.2. Effect of CO_2 on fruit quality and ripening of tomatoes

The CO_2 treatment had no significant effect on the fruit eating quality based on SSC or TA. Nevertheless, the pH of the treated fruits was higher than that of the untreated fruits after storage at 4°C for 14 d and after transfer to 20°C for 4–8 d (Table 1). Fruit firmness decreased with storage time; however, tomatoes treated with 30% or 60% CO_2 were significantly firmer than the control tomatoes when they were transferred to 20°C for 8 d after cold storage (Table 1). Notably, a delay in the decrease in firmness was only observed during shelf-life storage and not during cold storage. Tomato belongs to the classification of climacteric fruit which follow a ripening pattern that is controlled by ethylene; therefore, ripening is delayed if ethylene production is inhibited [13]. As shown in Figure 1B, ethylene production in CO_2 -treated tomatoes during shelf-life storage at 20°C was lower than that in the control. The data suggested that the lower ethylene production in CO_2 -treated tomatoes led to a delay in tomato softening. There were no significant differences between 30% and 60% CO_2 treatments in terms of firmness, SSC, and TA.

Table 1. Firmness, solid soluble content (SSC), titratable acidity (TA), and pH of tomatoes treated or not treated with CO₂ and stored for 0 d, 7 d, or 14 d at 4°C or stored at 4°C for 14 d followed by 8 d at 20°C (14 + 8 d).

Treatment	0 d	7 d	14 d	14 + 8 d
<i>Firmness (N)</i>				
Control	19.83±0.41 Aa ¹	16.56±0.18 Ab	13.67±0.34 Ac	6.54±0.38 Bd
30 % CO ₂	19.94±0.47 Aa	15.88±1.23 Ab	13.53±0.28 Ac	8.07±0.50 Ad
60 % CO ₂	20.42±0.67 Aa	17.84±0.70 Ab	13.19±0.57 Ac	7.94±0.51 Ad
<i>SSC (%)</i>				
Control	4.28±0.08 Aa	4.46±0.04 Aa	4.48±0.02 Aa	4.48±0.07 Aa
30 % CO ₂	4.42±0.10 Aa	4.44±0.07 Aa	4.46±0.07 Aa	4.50±0.06 Aa
60 % CO ₂	4.26±0.07 Ab	4.50±0.03 Aa	4.38±0.04 Aab	4.40±0.04 Aa
<i>TA (%)</i>				
Control	1.07±0.01 Aa	1.05±0.02 Aa	0.79±0.05 Ab	0.66±0.01 Ac
30 % CO ₂	0.94±0.02 Aa	0.99±0.02 ABa	0.85±0.03 Ab	0.68±0.01 Ac
60 % CO ₂	1.08±0.01 Aa	0.98±0.02 Ab	0.83±0.02 Ac	0.65±0.02 Ad
<i>pH</i>				
Control	3.99±0.02 Ab	3.97±0.01 Bb	3.95±0.03 Ab	4.32±0.04 Aa
30 % CO ₂	3.99±0.02 Ac	4.01±0.02 ABbc	4.07±0.04 Ab	4.34±0.02 Aa
60 % CO ₂	3.93±0.02 Ac	4.05±0.02 Ab	4.05±0.04 Ab	4.40±0.02 Aa

¹ Values are the means of 15 replicate samples ± standard errors. The same capital letter within each column, or the same small letter within each row, indicates means that are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

Pre-storage short-term exposure to CO₂ treatment delayed the ripening of tomato stored at 4°C for 14 d, even after they were transferred to 20°C for 8 d. Color is one of the most important visual attributes in the ripening index of tomato [17]. Fruits treated with 30% and 60% CO₂ had a lower a* than untreated fruits during cold storage for 14 d but not after transfer to 20°C (Figures 2A and B). The color change during the ripening of the tomato fruit is due to the degradation of chlorophyll coupled with the synthesis of different types of anthocyanins and the accumulation of carotenoids such as β-carotene, xanthophyll esters, xanthophylls, and lycopene. In particular, lycopene accumulation is correlated with the tomato fruit [23]. To validate these results and determine the influence of CO₂ on ripening, we investigated the carotenoid content of tomatoes during the storage period. As expected, tomatoes treated with CO₂ showed lower lycopene contents than untreated tomatoes. In contrast, CO₂ treatment CO₂ enhanced the beta-carotene content of tomatoes at the beginning of storage, whereas the lutein content decreased while the storage time increased (Table 2). Under cold storage, the CO₂-treated tomatoes showed significantly lower lycopene contents than the control. However, beta-carotene and lutein contents were higher in the tomatoes treated with CO₂ than in the untreated control. These results are similar to those obtained by Park et al. [17], who showed that cold storage inhibited lycopene synthesis in tomatoes. Under shelf-life conditions, lycopene content increased dramatically in coordination with color change following exposure to CO₂ treatment (Figure 2B). These results suggest that CO₂ treatment delayed ripening and extended the shelf life of tomatoes and that these effects were synergistic under cold storage conditions.

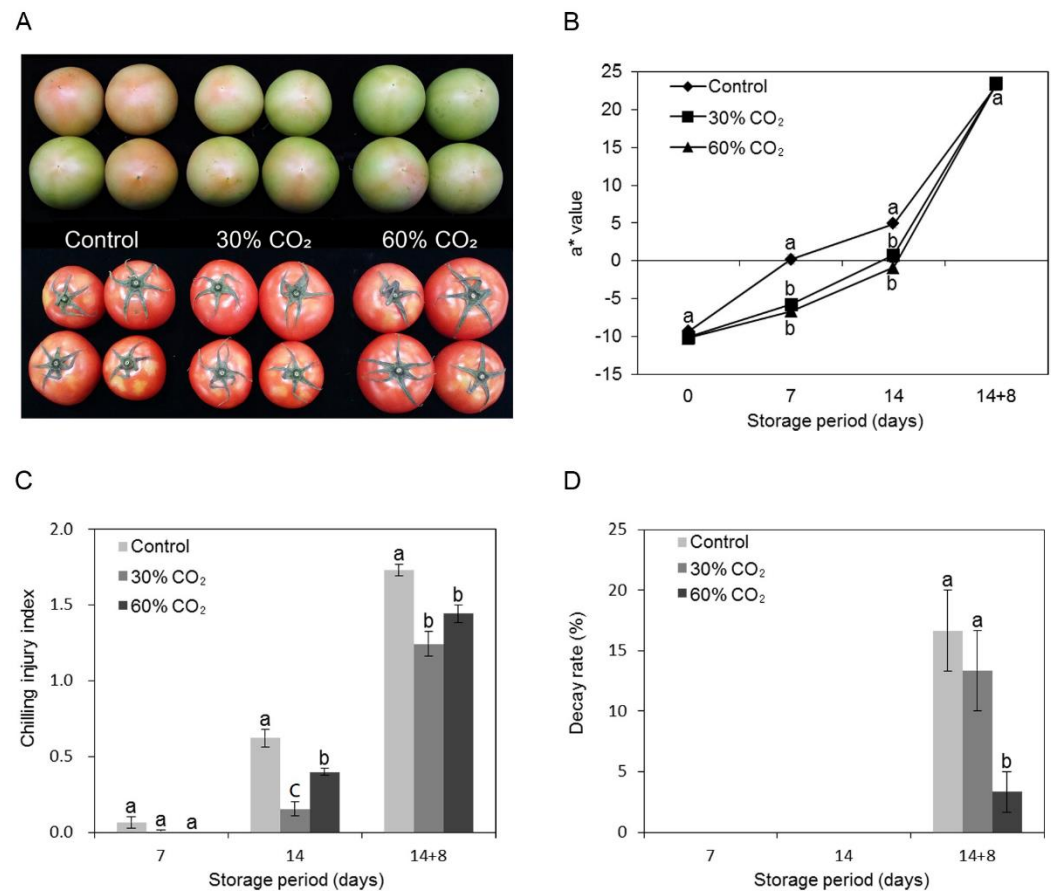


Figure 2. Effect of short-term CO₂ treatment on tomato quality (A) Photographs of CO₂-treated and untreated tomatoes taken after 7 d of cold storage at 4°C (upper) and after 14 d at 4°C followed by 8 d at 20°C (lower). (B) Changes in the skin color *a** value (redness) of CO₂-treated or untreated tomatoes during storage at 4°C for 14 d and then transferred to 20°C for another 8 d (14 + 8). Values are the means of 15 replicate samples \pm SE. (C) Chilling injury index of tomato. (D) Decay rate of tomato. Data are shown as the mean \pm SE of three replicates. Untreated tomatoes were used as the control.

Table 2. Changes in the carotenoid content of tomatoes treated or not treated with CO₂ and stored for 0 d, 7 d, or 14 d at 4°C or stored for 14 d at 4°C followed by 8 d at 20°C.

Treatment	0 d	7 d	14 d	14 + 8 d
<i>Lutein (mg kg⁻¹)</i>				
Control	27.84 \pm 4.16 Aa	24.45 \pm 2.95 Ba	10.94 \pm 1.32 Ab	8.85 \pm 0.36 Ab
30% CO ₂	32.45 \pm 1.95 Aa	34.94 \pm 4.55 ABa	11.88 \pm 2.06 Ab	8.55 \pm 0.32 Ab
60% CO ₂	33.80 \pm 1.85 Aa	36.25 \pm 0.01 Aa	11.60 \pm 0.63 Ab	8.61 \pm 0.64 Ab
<i>Lycopene (mg kg⁻¹)</i>				
Control	5.05 \pm 0.43 Ab	4.82 \pm 0.69 Ab	10.64 \pm 1.79 Ab	39.56 \pm 3.87 Aa
30% CO ₂	6.64 \pm 1.54 Ab	4.74 \pm 0.72 Ab	6.45 \pm 1.48 ABb	26.88 \pm 0.73 Ba
60% CO ₂	6.21 \pm 0.12 Ab	3.37 \pm 0.44 Ab	4.72 \pm 0.18 Bb	26.57 \pm 2.21 Ba
<i>Beta-carotene (mg kg⁻¹)</i>				
Control	15.06 \pm 2.84 Aa	9.64 \pm 1.88 Bab	7.38 \pm 0.80 Ab	10.24 \pm 0.18 Aab
30% CO ₂	14.67 \pm 0.79 Aab	15.92 \pm 2.28 Aa	9.73 \pm 2.54 Abc	8.36 \pm 0.29 Ac
60% CO ₂	17.02 \pm 2.30 Aa	16.26 \pm 0.64 Aa	7.51 \pm 0.97 Ab	9.33 \pm 0.91 Ab

¹ Values are the means \pm standard errors of 15 replicate samples. The same capital letter within each column, or the same small letter within each row, indicates means that are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

3.3. Effect of CO₂ on chilling injury and decay of tomatoes

CI symptoms were observed in the control fruits after 7 d of storage at 4°C and increased after the treatment for 14 d at 4°C, followed by 8 d at 20°C (Figure 2C). A previous report showed that mature, green tomatoes stored below 12.5°C for longer than two weeks showed CI symptoms such as surface pitting and increased fungal growth during subsequent ripening at ambient temperatures [24]. However, treatment group with 30% or 60% CO₂ showed significantly reduced CI symptoms (based on pitting) than the control fruits at 14 d at 4°C and 14 d at 4°C, followed by 8 d at 20°C (Figure 2C). These findings indicate that CO₂ treatment suppressed CI. In particular, 30% CO₂ treatment prevented CI symptoms, such as surface pitting, more effectively than treatment with 60% CO₂.

The decay rate increased dramatically in fruits transferred to shelf-life conditions at 20°C after cold storage, whereas the group exposed to 60% CO₂ had a significantly reduced decay rate (Figure 2D). Therefore, 30% or 60% CO₂ treatments can be applied to reduce the decay rate during the cold storage of tomatoes.

3.4. RNA-Seq and functional categorization of CO₂-responsive genes

To understand how CO₂ treatment regulated the physiological and biochemical modifications related to CI, a comparative transcriptomic analysis was performed using the pericarp tissue from CO₂-treated and untreated tomatoes and/or cold storage. Large number of DEGs were identified from the CO₂ treatments on day 0. The complete details of DEGs from all comparisons, including Venn diagrams, are provided in Figure S1. The heat map revealed dramatic changes after the CO₂ treatments, which separated the control tomatoes at each storage time point (Figure S2).

To provide a functional overview of the DEGs between the CO₂-treated and untreated groups, DAVID analysis was performed using all the CO₂-responsive genes. The GO terms annotated for the DEGs belonged to 21 functional groups, including cellular components, biological processes, and molecular functions (Figure S3), implying that these DEGs are functionally involved in diverse physiological processes. In the KEGG database, the most abundant pathway (lowest *p*) was the biosynthesis of secondary metabolites (sly01110) (Figure 3A).

Conserved domains in the DEGs were further identified, in particular, the most abundant domain was the *ethylene response factor (ERF)* domain (Figure 3B). Therefore, we examined the effects of CO₂ treatment on ethylene-related genes. Gene expression analysis showed that the ethylene signaling-related genes *ERF1*, *ERF2*, and *ERF4* were upregulated upon CO₂ treatment at day 0. In contrast, the ethylene synthesis-related genes *1-aminocyclopropane-1-carboxylate synthase 4 (ACS4)* were significantly downregulated during cold storage after CO₂ treatment (Figure 4). However, CO₂ treatment and cold storage, followed by shelf-life conditions, downregulated the ethylene response factor genes *ERF107*. These results suggest that CO₂ treatment reduced ethylene synthesis and subsequently enhanced ethylene signaling to extend the shelf life of tomatoes.

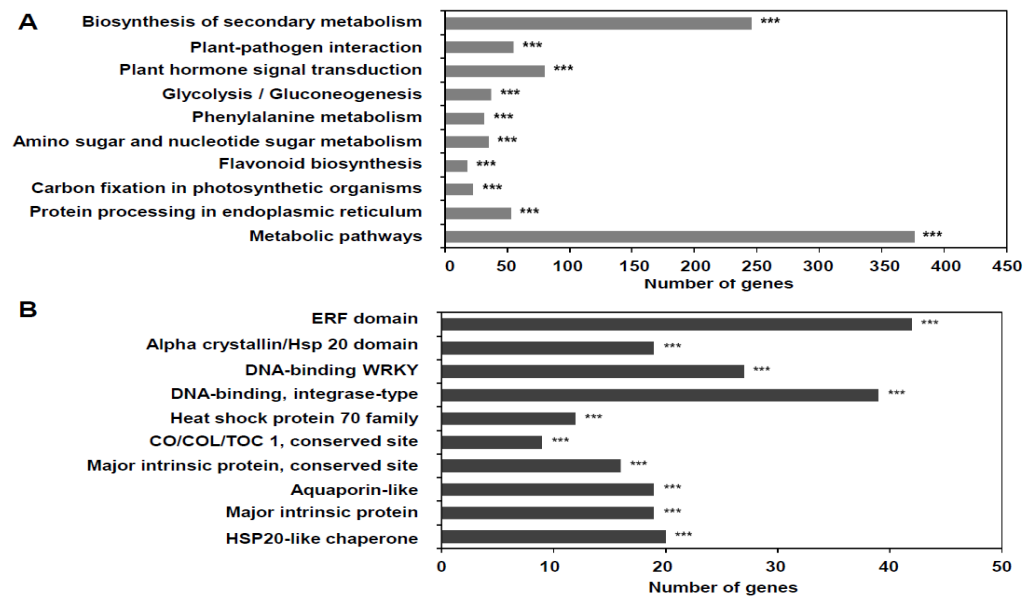


Figure 3. Global analysis of DEGs in CO₂-treated tomatoes. (A) KEGG pathway enrichment analysis of the DEGs. (B) InterPro domain analysis of DEGs. Samples were obtained from untreated (control) and CO₂-treated tomatoes. Analyses were performed using DAVID v.6.8. *** represents $p \leq 0.001$.

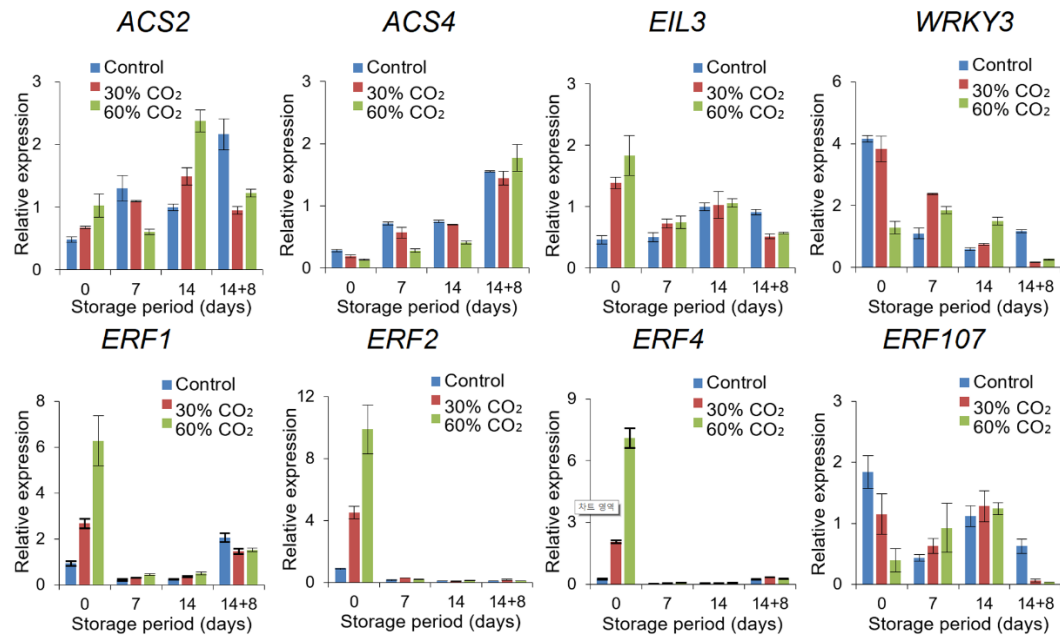


Figure 4. Quantitative real-time-PCR validation of DEGs identified by RNA-Seq analysis. The expression of selected DEGs, including the ethylene-related genes and WRKY, examined via qRT-PCR. Samples were obtained from tomatoes treated or not treated with CO₂ after 0 d, 7 d, and 14 d at 4°C and after 14 d at 4°C followed by 8 d at 20°C (14 + 8 d). The bar represents the mean \pm standard error of three biological replicates

3.5. Primary metabolite profiling for pathway analysis

Metabolites of untreated and CO₂-treated tomatoes during postharvest storage for the treatment including 0 d and 14 d at 4°C and the treatment including 14 d at 4°C fol-

lowed by 8 d at 20°C were analyzed by GC-MS. The water-soluble metabolites are presented based on the KEGG pathway (Figure 5). To analyze KEGG pathway using MetaboAnalyst, control and 30% CO₂ treatment at above time points were selected to characterize the treatment effect. There was no considerable difference between 7 d and 14 d at 4°C. Thus, metabolites from 7 d at 4°C was not included here. The relative concentrations of sugar, valine, and glutamic acid were significantly decreased after 30% CO₂ treatment on day 0 ($p < 0.01$). Similarly, the relative concentrations of phenylalanine, tyrosine, aspartic acid, and lysine were significantly decreased after 30% CO₂ treatment on day 0 ($p < 0.05$). The relative concentration of sucrose was significantly increased in the 30% CO₂ treatment on day 14 at 4°C ($p < 0.05$). Meanwhile, the relative concentrations of alanine, valine, lysine, glutamic acid, serine, glycine, and tyrosine were significantly increased in the 30% CO₂ treatment at 4°C for 14 d, followed by 8 d at 20°C (14 + 8 d) ($p < 0.05$). Relative concentrations of sucrose and glutamine were significantly increased after 30% CO₂ treatment at 4°C for 14 d, followed by 8 d at 20°C (14 + 8 d) ($p < 0.05$), whereas the relative concentration of fructose was significantly decreased after 30% CO₂ treatment at 4°C for 14 d, followed by 8 d at 20°C (14 + 8 d) ($p < 0.05$).

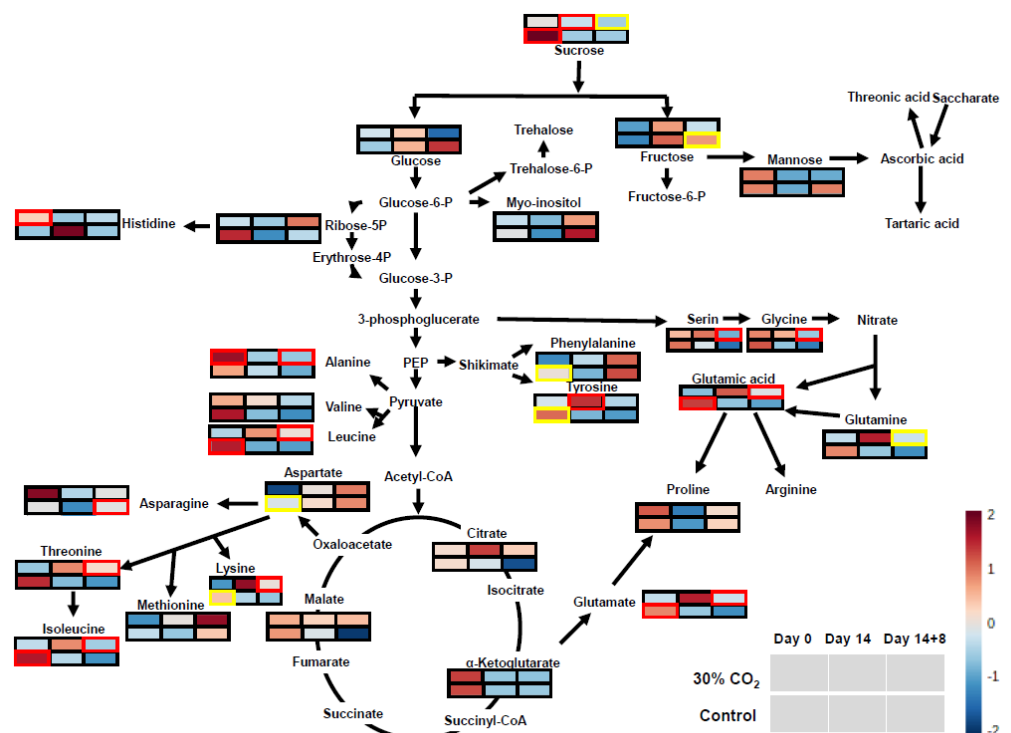


Figure 5. Map of metabolites involved in sugar, amino acid, and TCA metabolite pathways. Map of metabolites in red and blue represent those upregulated and downregulated, respectively, by no treatment and CO₂ treatment. Red ($p = 0.01$) and yellow ($p = 0.05$) boxes represent significantly different p -values by Tukey's HSD. Their relative levels of expression in the control and 30% CO₂ treatment groups at days 0, 14, and 14 + 8 are shown as a heat map.

PLS-DA was conducted using Pareto scaling from GC-MS data. Regarding the PLS-DA score plot (Figure 6A) of the 30% CO₂ treatment at 4°C for 14 d followed by a period of 8 d at 20°C (14 + 8 d), component 1 explained 51.7% of the total variance, and component 2 explained 15.9% of the total variance. The two treatments were tightly clustered and separated from each other on a score plot without overlapping of the 95% confidence intervals. The PLS-DA loading plot (Figure 6B) of the 30% CO₂ treatment at 4°C for 14 d followed by 8 d at 20°C (14 + 8 d) showed that most metabolites were located on the left.

Threonine, serine, tyrosine, glycine, beta-aminoisobutyric acid, valine, and leucine had high variable importance of projection (VIP) values (Figure 6C), indicating that these metabolites are useful biomarkers of tomato during postharvest storage for 14 d at 4°C, followed by 8 d at 20°C. With regard to the PLS-DA score plot (Figure S4A) of the 60% CO₂ treatment at 4°C for 14 d followed by 8 d at 20°C (14 + 8 d), component 1 explained 54% of the total variance, and component 2 explained 15% of the total variance. Additionally, the PLS-DA loading plot (Figure S4B) of the 60% CO₂ treatment at 4°C for 14 d followed by 8 d at 20°C (14 + 8 d) showed that most metabolites were located on the left. Tyrosine, leucine, aspartic acid, sucrose, and proline had high VIP values (Figure S4C), indicating that these metabolites are biomarkers of tomato during postharvest storage for 14 d at 4°C, followed by 8 d at 20°C (14+8 d).

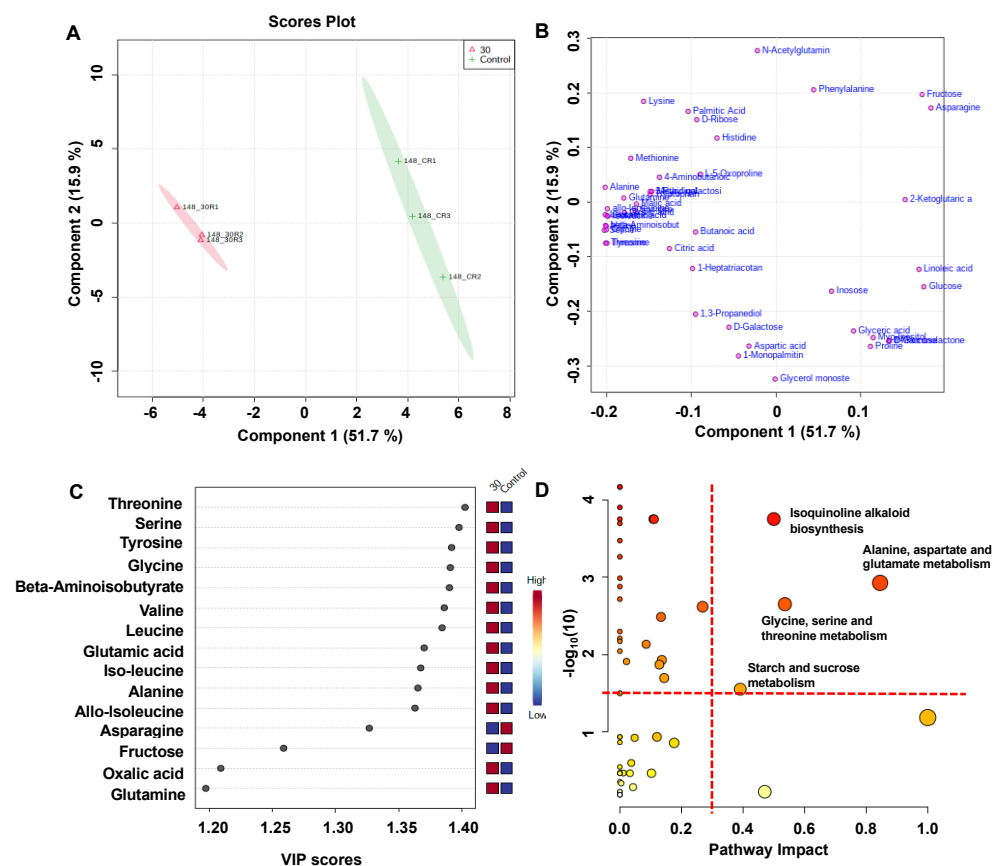


Figure 6. Partial least squares-discriminant analysis (PLS-DA) score plot (A) and loading plot (B) derived from GC-MS data of untreated and 30% CO₂-treated tomatoes during postharvest storage for 14 d at 4°C, followed by 8 d at 20°C (14 + 8 d). PLS-DA variable importance of projection (VIP) score (C) derived from GC-MS data of untreated and 30% CO₂-treated tomatoes during postharvest storage for 14 d at 4°C, followed by 8 d at 20°C (14 + 8 d). Top 15 VIP scores of metabolites characterized by VIP scores (>1.238) in the KEGG pathway analysis from untreated and 30% CO₂-treated tomatoes (D) during postharvest storage for 14 d at 4°C, followed by 8 d at 20°C (14 + 8 d). The named pathways in bold represent the significantly changed metabolism characterized by $-\log_{10}(p)$ value (>1.5) and impact value (0.3).

Pathway analysis was performed using metabolites from the 30% (Figure 6D) and 60% (Figure S4D) CO₂ treatments at 4°C for 14 d, followed by 8 d at 20°C (14 + 8 d). Three metabolic pathways (isoquinoline alkaloid biosynthesis; alanine, aspartate, and glutamate metabolism; and glycine, serine, and threonine metabolism) between the CO₂-treated

groups and the untreated group significantly changed metabolisms and were characterized by $-\log_{10}(p)$ value (>1.5) and impact value (0.3).

4. Discussion

4.1. Role of CO₂ treatment in the response of tomato fruit to low temperature

Although CO₂ treatments have a beneficial effect on the postharvest quality of several crops, short-term pretreatment with CO₂ has not been applied to tomato as a postharvest technology. As a result, the mechanism underlying its beneficial effects remains unknown. In the present study, we found that short-term CO₂ pretreatment delayed ripening and reduced CI symptoms, consequently extending the shelf life of tomatoes. The ripening process in tomato involves a complex and coordinated series of changes in pigmentation, flavor, texture, and aroma, resulting from physiological and biochemical activities. However, cold storage is known to inhibit lycopene synthesis [17]. In the present study, short-term CO₂ treatment had a synergistic effect on the inhibition of lycopene development under cold storage, as revealed by the lower lycopene content in the CO₂-treated groups than the untreated tomatoes (Table 2). Both the treatment with high levels of CO₂ and the act of cold storage inhibited lycopene development, resulting in a low a^* value (Figure 2). Treatment with high concentrations of CO₂ blocked or delayed ripening by suppressing ripening-related gene expression [25]. Rugkong et al. [26] reported that uneven ripening in cold-stored tomatoes was related to the downregulation of genes associated with ethylene biosynthesis and signaling, which was reflected in the reduced ethylene production and lycopene accumulation observed in the experiment. In the present study, ethylene production was not significantly different between the CO₂-treated and untreated tomatoes under cold-storage conditions (Figure 1). Nevertheless, it should be noted that evident changes in ethylene production due to CO₂ treatment are difficult to observe because cold storage restricts ethylene production. Hence, CO₂-induced delays in ripening have a synergistic relationship with cold storage.

However, short-term CO₂-treated tomatoes maintained significantly more firmness than the untreated control fruits under shelf-life conditions after cold storage. Under shelf-life conditions after cold storage, the CO₂-treated tomatoes showed less ethylene production than the control, suggesting its role in fruit softening. A previous study found that CO₂ treatment increased firmness in peach, suggesting that cell wall-degrading enzyme activity is altered by exposure to high CO₂ concentrations [12]. High CO₂ levels also influence cell-wall calcium binding, thereby increasing fruit firmness [27]. Furthermore, a recent report showed that CO₂ treatment delayed cell wall degradation, thus maintaining the integrity of the middle lamella in strawberry and downregulating the level of the cell degradation enzyme, pectin esterase [10]. An increase in fruit firmness resulting from postharvest CO₂ treatment occurs primarily through calcium-mediated pectin polymerization [28].

The CO₂-treated tomatoes also showed a lower CI index than the control (Figure 2C). CI is related to increased membrane permeability, increased leakage of ions from cells into intercellular spaces within tissues [2], and ultrastructural changes in the membrane [29]. Moreover, in the present study, functional analysis of DEGs showed that CO₂-responsive genes were most significantly involved in the integral components of the plasma membrane, the regulation of defense responses, and cell wall biogenesis (Figure S2).

4.2. CO₂-induced global transcriptional changes

Comparative transcriptome analysis, performed to investigate the mechanism by which CO₂ treatment affects fruit quality, showed dramatic transcriptomic changes between the CO₂-treated and untreated tomatoes. Interestingly, functional analysis showed

that the major domain of the DEGs was the ERF domain (Figure 3B). The ERFs is known to participate in the last step of the ethylene signal transduction pathway and play important roles in the fruit ripening process and abiotic stress response [30,31]. ERF proteins play an important role in the cold response by regulating the expression of downstream stress-related genes [32]. In tomato, 77 ERFs have been identified, 19 of which are related to ripening [33]. Most tomato ERFs, such as *LeERF*, *Pti*, and *JERF*, are responsive to environmental stresses, including low temperature, wounding, and salinity [27][34,35]. The ERFs identified in the present study responded differently at each time point. While *ERF1*, *ERF2*, and *ERF4* were upregulated by CO₂ treatment at day 0, *ERF107* were downregulated under shelf-life conditions after cold storage (14 + 8 d) (Figure 4). Romero et al.[36] reported that ERF genes play a role in the beneficial effect of high CO₂ levels on the maintenance of table grape quality during storage at low temperatures, while *VviERF2c* may play a role in modulating *PR* gene expression. ERFs involved in CI are reduced by methyl jasmonate, suggesting that *ERF1* plays a role in regulating CI [37]. The present study suggests that CO₂ treatment triggered ethylene signaling, especially involving ERFs that regulate cold stress, and reduced CI in tomato.

For tomato fruit ripening, ethylene synthesis, perception, and signaling are very important events because tomato is a climacteric fruit that shows increased ethylene production at, or just before, the onset of ripening and requires ethylene to complete the ripening process [38]. It is possible that ERFs regulate ethylene synthesis to maintain tomato fruit quality by controlling the ripening process. The ethylene biosynthetic pathway comprises two steps. First, S-adenosylmethionine is converted into 1-aminocyclopropane-1-carboxylate (ACC) by the rate-limiting enzyme, ACC synthase (ACS). ACC is then converted to ethylene by ACC oxidase (ACO) [39]. Although ACO activity is not a rate-limiting step, certain ACOs are ethylene-inducible, and their silencing prevents ethylene synthesis and ripening [39]. ACS is a key enzyme in ethylene biosynthesis and in the regulation of the transition from system-1 to system-2 ethylene synthesis in tomato. Previous RNA-Seq analysis using the “Micro Tom” variety of tomato showed that chilling blocked the second step of ethylene biosynthesis [1]. Another study revealed that the regulation of *TERF2/LeERF2* is associated with enhanced freezing tolerance in tobacco and tomato through ethylene biosynthesis [32].

LeACS2 and *LeACS4* expression levels increase during tomato fruit ripening [40]. The genes, *LeACO1* and *LeACO4* encode the last enzyme in ethylene biosynthesis [41]. In the present study, the ethylene synthesis genes *ACS4* were significantly downregulated by CO₂ treatment (Figure 4). This suggests that delayed ripening occurs via the blocking of ethylene synthesis by both CO₂ treatment and cold storage. Our findings suggest that CO₂ treatment reduced CI symptoms and delayed ripening by regulating both ethylene synthesis and ethylene signaling, especially involving ERFs, which, in turn, controls other downstream factors. However, further research is required to elucidate the link between ERFs and downstream factors that control CI occurrence.

4.3. Effect of CO₂ treatment on tomato metabolite and quality

The tomatoes that underwent short-term CO₂ treatment showed significant changes in the metabolites involved in starch and sucrose metabolism ($p = 0.03$, impact = 0.39). In the present study, the sucrose concentration of tomatoes treated with CO₂ over a short term was significantly higher than that of untreated tomatoes. A previous study reported that acid invertase activity significantly affects the ratio between sucrose and monosaccharides, including glucose and fructose [42]. In our study, the soluble acid invertase activity of short-term CO₂-treated tomatoes might have decreased along with a reduction in respiration rate (glycolysis). This result also consistent with the highly expressed glycolysis/gluconeogenesis from KEGG pathway enrichment analysis based on transcriptome (Figure 3). We also found that the levels of malic acid and citric acid in short-term CO₂-treated tomatoes were not significantly different from those in the untreated ones.

Sangwanangkul et al.[13] reported several organic acid concentration changes in cherry tomatoes after 20% and 60% CO₂ treatment. Although a significant decay rate was observed in the 60% CO₂ treatment at 12°C, there was no specific organic acid trend observed after the CO₂ treatment in the study. According to experiment of Centeno et al., [43] modified malate metabolism in tomatoes significantly affected on sugar and starch metabolism. Consequently, the modified malate metabolism changed resistance to *Botrytis cinerea* through increased wrinkling. However, we did not observe any significant changed in malate metabolism. Taken together, the improved quality of tomato fruits may be the synergistic effect of CO₂ pretreatment prior to cold storage.

The four metabolisms were affected in significantly different ways by CO₂ treatment. Isoquinoline alkaloid biosynthesis and alanine, aspartate, and glutamate metabolisms have been reported as drought stress-related metabolisms from drought stress experiments [44,45]. Water loss during postharvest storage may be recognized as associated with drought stress. Glutamate is the first amino acid related to nitrogen fixation and is decreased by drought stress because plants cannot take up nitrogen fertilizer from the soil under drought stress. During postharvest storage, the fruit cannot obtain exogenous nitrogen while maintaining cellular activity, which includes consuming energy and converting compounds. Thus, control tomatoes with higher levels of respiration could have spent amino acids rapidly, resulting in differences in alanine, aspartate, and glutamate metabolisms. After 14 days of cold storage and 8 days of room temperature shelf-life conditions, CO₂-treated tomatoes showed higher concentrations of 10 different amino acids than the control tomatoes. These included: serine, glycine, glutamate, glutamine, tyrosine, alanine, leucine, lysine, threonine, and isoleucine. Treatment with CO₂ significantly changed amino acid and sugar concentrations after cold and shelf-life conditions, indicating that CO₂-treated tomatoes might have a better taste than untreated tomatoes.

Biosynthesis of secondary metabolism was highly expressed from KEGG pathway enrichment analysis based on transcriptome (Figure 3A). The result may be related with carotenoid biosynthesis since 60% CO₂ treatment significantly delayed lycopene biosynthesis at 14 d and 14 d and additional 8 day of shelf-life condition. Plant-pathogen interaction is mostly related with secondary metabolism. Flavonoid biosynthesis is starting from shikimate pathway based on phenylalanine as substrate. Thus, the selected DEGs complementarily explained each other. Previous study [46] reported that the most abundant flavonoids in tomatoes were quercetin and rutin. These flavonoids were gradually increased from breaker stage to the pink or light red stages, depending on the cultivar.

5. Conclusions

In summary, integrated transcriptome and metabolome profiling provided the basic physiochemical information underlying the response of tomato to CO₂ treatment. This suggests that CO₂ treatment delayed fruit ripening by regulating carbohydrate metabolism and ethylene-related genes. Therefore, our findings will help in developing strategies to reduce CI symptoms and extend the shelf life of other subtropical crops.

Supplementary Materials: Table S1: Sequences of primers used for qRT-PCR, Figure S1: Venn diagram showing DEGs in CO₂-treated versus untreated tomatoes at each time point, Figure S2: GoTerm analysis of DEGs, Figure S3: Expression profiling of DEGs involved in ethylene signaling and synthesis. Figure S4: Partial least squares-discriminant analysis (PLS-DA) score plot (A) and loading plot (B) derived from GC-MS data of untreated and 30% CO₂-treated tomatoes during post-harvest storage for 14 d at 4°C, followed by 8 d at 20°C (14 + 8 d).

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References

1. Cruz-Mendivil, A.; López-Valenzuela, J.A.; Calderón-Vázquez, C.L.; Vega-García, M.O.; Reyes-Moreno, C.; Valdez-Ortiz, A. Transcriptional changes associated with chilling tolerance and susceptibility in 'Micro-Tom' tomato fruit using RNA-Seq. *Postharvest biology and technology* **2015**, *99*, 141-151.
2. Saltveit, M.; Morris, L. Overview on chilling injury of horticultural crops. *Chilling injury of horticultural crops* **1990**, *1*, 3-15.
3. Vega-García, M.O.; López-Espinoza, G.; Ontiveros, J.C.; Caro-Corrales, J.J.; Vargas, F.D.; López-Valenzuela, J.A. Changes in protein expression associated with chilling injury in tomato fruit. *Journal of the American Society for Horticultural Science* **2010**, *135*, 83-89.
4. Lauxmann, M.A.; Brun, B.; Borsani, J.; Bustamante, C.A.; Budde, C.O.; Lara, M.V.; Drincovich, M.F. Transcriptomic profiling during the post-harvest of heat-treated Dixiland Prunus persica fruits: common and distinct response to heat and cold. *PloS one* **2012**, *7*, e51052.
5. Arnal, L.; Del Río, M. Removing astringency by carbon dioxide and nitrogen-enriched atmospheres in persimmon fruit cv. "Rojo brillante". *Journal of Food Science* **2003**, *68*, 1516-1518.
6. Blanch, M.; Rosales, R.; Mateos, R.; Perez-Gago, M.B.; Sanchez-Ballesta, M.T.; Escibano, M.I.; Merodio, C. Effects of high CO₂ levels on fermentation, peroxidation, and cellular water stress in *Fragaria vesca* stored at low temperature in conditions of unlimited O₂. *Journal of agricultural and food chemistry* **2015**, *63*, 761-768.
7. Dong, T.; Shi, J.; Jiang, C.-Z.; Feng, Y.; Cao, Y.; Wang, Q. A short-term carbon dioxide treatment inhibits the browning of fresh-cut burdock. *Postharvest Biology and Technology* **2015**, *110*, 96-102.
8. Larsen, M.; Watkins, C.B. Firmness and aroma composition of strawberries following short-term high carbon dioxide treatments. *HortScience* **1995**, *30*, 303-305.
9. Pérez, A.G.; Sanz, C. Effect of high-oxygen and high-carbon-dioxide atmospheres on strawberry flavor and other quality traits. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2370-2375.
10. Bang, J.; Lim, S.; Yi, G.; Lee, J.G.; Lee, E.J. Integrated transcriptomic-metabolomic analysis reveals cellular responses of harvested strawberry fruit subjected to short-term exposure to high levels of carbon dioxide. *Postharvest Biology and Technology* **2019**, *148*, 120-131.
11. Min, T.; Yin, X.-r.; Shi, Y.-n.; Luo, Z.-r.; Yao, Y.-c.; Grierson, D.; Ferguson, I.B.; Chen, K.-s. Ethylene-responsive transcription factors interact with promoters of ADH and PDC involved in persimmon (*Diospyros kaki*) fruit de-astringency. *Journal of Experimental Botany* **2012**, *63*, 6393-6405.
12. Chang, E.-H.; Lee, J.-S.; Kim, J.-G. Cell wall degrading enzymes activity is altered by high carbon dioxide treatment in postharvest 'Mihong' peach fruit. *Scientia horticulturae* **2017**, *225*, 399-407.
13. Sangwanankul, P.; Bae, Y.-S.; Lee, J.-S.; Choi, H.-J.; Choi, J.-W.; Park, M.-H. Short-term pretreatment with high CO₂ alters organic acids and improves cherry tomato quality during storage. *Horticulture, Environment, and Biotechnology* **2017**, *58*, 127-135.
14. Ezz, T.M.; Ritenour, M.A.; Brecht, J.K. Hot Water and Elevated CO₂ Effects on Proline and Other Compositional Changes in Relation to Postharvest Chilling Injury of Marsh'Grapefruit. *Journal of the American Society for Horticultural Science* **2004**, *129*, 576-582.

15. Bertolini, P.; Lanza, G.; Tonini, G. Effects of pre-storage carbon dioxide treatments and storage temperatures on membranosis of 'Femminello comune' lemons. *Scientia horticulturae* **1991**, *46*, 89-95.
16. Serrano, M.; Pretel, M.T.; Martínez-Madrid, M.C.; Romojaro, F.; Riquelme, F. CO₂ treatment of zucchini squash reduces chilling-induced physiological changes. *Journal of Agricultural and Food Chemistry* **1998**, *46*, 2465-2468.
17. Park, M.-H.; Sangwanangkul, P.; Choi, J.-W. Reduced chilling injury and delayed fruit ripening in tomatoes with modified atmosphere and humidity packaging. *Scientia Horticulturae* **2018**, *231*, 66-72.
18. Zarei, A.; Zamani, Z.; Mousavi, A.; Fatahi, R.; Alavijeh, M.K.; Dehsara, B.; Salami, S.A. An effective protocol for isolation of high-quality RNA from pomegranate seeds. *Asian Aust J Plant Sci Biotechnol* **2012**, *6*, 32-37.
19. Perte, M.; Kim, D.; Perte, G.M.; Leek, J.T.; Salzberg, S.L. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature protocols* **2016**, *11*, 1650.
20. Caraux, G.; Pinloche, S. PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics* **2005**, *21*, 1280-1281.
21. Lisc, J.; Schauer, N.; Kopka, J.; Willmitzer, L.; Fernie, A.R. Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature protocols* **2006**, *1*, 387.
22. Kim, H.-J.; Ku, K.M.; Choi, S.; Cardarelli, M. Vegetal-derived Biostimulant Enhances Adventitious Rooting in Cuttings of Basil, Tomato, and Chrysanthemum via Brassinosteroid-mediated Processes. *Agronomy* **2019**, *9*, 74, doi:10.3390/agronomy9020074.
23. Cox, S.; Stushnoff, C.; Sampson, D. Relationship of fruit color and light exposure to lycopene content and antioxidant properties of tomato. *Canadian Journal of Plant Science* **2003**, *83*, 913-919.
24. Suslow, T.; Cantwell, M. Tomato: Recommendations for maintaining postharvest quality. *Produce Facts. Davis: Postharvest Technology Research & Information Center* **2009**.
25. Rothan, C.; Duret, S.; Chevalier, C.; Raymond, P. Suppression of ripening-associated gene expression in tomato fruits subjected to a high CO₂ concentration. *Plant Physiology* **1997**, *114*, 255-263.
26. Rugkong, A.; McQuinn, R.; Giovannoni, J.J.; Rose, J.K.; Watkins, C.B. Expression of ripening-related genes in cold-stored tomato fruit. *Postharvest Biology and Technology* **2011**, *61*, 1-14.
27. Wang, M.H.; Kim, J.G.; Ahn, S.E.; Lee, A.Y.; Bae, T.M.; Kim, D.R.; Hwang, Y.S. Potential role of pectate lyase and Ca²⁺ in the increase in strawberry fruit firmness induced by short-term treatment with high-pressure CO₂. *Journal of food science* **2014**, *79*, S685-S692.
28. Hwang, Y.S.; Min, J.H.; Kim, D.Y.; Kim, J.G.; Huber, D.J. Potential mechanisms associated with strawberry fruit firmness increases mediated by elevated pCO₂. *Horticulture, Environment, and Biotechnology* **2012**, *53*, 41-48.
29. Yang, T.; Chaudhuri, S.; Yang, L.; Du, L.; Poovaiah, B. A calcium/calmodulin-regulated member of the receptor-like kinase family confers cold tolerance in plants. *Journal of Biological Chemistry* **2010**, *285*, 7119-7126.
30. Yin, X.-r.; Allan, A.C.; Xu, Q.; Burdon, J.; Dejnopratt, S.; Chen, K.-s.; Ferguson, I.B. Differential expression of kiwifruit ERF genes in response to postharvest abiotic stress. *Postharvest biology and technology* **2012**, *66*, 1-7.
31. Liu, M.; Pirrello, J.; Kesari, R.; Mila, I.; Roustan, J.P.; Li, Z.; Latché, A.; Pech, J.C.; Bouzayen, M.; Regad, F. A dominant repressor version of the tomato S1-ERF. B3 gene confers ethylene hypersensitivity via feedback regulation of ethylene signaling and response components. *The Plant Journal* **2013**, *76*, 406-419.
32. Zhang, Z.; Huang, R. Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. *Plant molecular biology* **2010**, *73*, 241-249.
33. Liu, M.; Gomes, B.L.; Mila, I.; Purgatto, E.; Peres, L.E.; Frasse, P.; Maza, E.; Zouine, M.; Roustan, J.-P.; Bouzayen, M. Comprehensive profiling of ethylene response factor expression identifies ripening-associated ERF genes and their link to key regulators of fruit ripening in tomato. *Plant Physiology* **2016**, *170*, 1732-1744.

34. Chen, G.; Hu, Z.; Grierson, D. Differential regulation of tomato ethylene responsive factor LeERF3b, a putative repressor, and the activator Pti4 in ripening mutants and in response to environmental stresses. *Journal of plant physiology* **2008**, *165*, 662-670.
35. Sharma, M.K.; Kumar, R.; Solanke, A.U.; Sharma, R.; Tyagi, A.K.; Sharma, A.K. Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. *Molecular Genetics and Genomics* **2010**, *284*, 455-475.
36. Romero, I.; Vazquez-Hernandez, M.; Escribano, M.I.; Merodio, C.; Sanchez-Ballesta, M.T. Expression profiles and DNA-binding affinity of five ERF genes in bunches of *Vitis vinifera* cv. Cardinal treated with high levels of CO₂ at low temperature. *Frontiers in plant science* **2016**, *7*, 1748.
37. Shin, S.-Y.; Park, M.-H.; Choi, J.-W.; Kim, J.-G. Gene network underlying the response of harvested pepper to chilling stress. *Journal of plant physiology* **2017**, *219*, 112-122.
38. Sun, Q.; Zhang, N.; Wang, J.; Zhang, H.; Li, D.; Shi, J.; Li, R.; Weeda, S.; Zhao, B.; Ren, S. Melatonin promotes ripening and improves quality of tomato fruit during postharvest life. *Journal of Experimental Botany* **2015**, *66*, 657-668.
39. Klee, H.J.; Giovannoni, J.J. Genetics and control of tomato fruit ripening and quality attributes. *Annual review of genetics* **2011**, *45*, 41-59.
40. Barry, C.S.; Llop-Tous, M.I.; Grierson, D. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology* **2000**, *123*, 979-986.
41. Chersicola, M.; Kladnik, A.; Tušek Žnidarič, M.; Mrak, T.; Gruden, K.; Dermastia, M. 1-Aminocyclopropane-1-carboxylate oxidase induction in tomato flower pedicel phloem and abscission related processes are differentially sensitive to ethylene. *Frontiers in plant science* **2017**, *8*, 464.
42. Klann, E.M.; Chetelat, R.T.; Bennett, A.B. Expression of acid invertase gene controls sugar composition in tomato (*Lycopersicon*) fruit. *Plant physiology* **1993**, *103*, 863-870.
43. Centeno, D.C.; Osorio, S.; Nunes-Nesi, A.; Bertolo, A.L.; Carneiro, R.T.; Araújo, W.L.; Steinhauser, M.-C.; Michalska, J.; Rohrmann, J.; Geigenberger, P. Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *The Plant Cell* **2011**, *23*, 162-184.
44. Jia, H.; Wang, L.; Li, J.; Sun, P.; Lu, M.; Hu, J. Physiological and metabolic responses of *Salix sinopurpurea* and *Salix suchowensis* to drought stress. *Trees* **2020**, *34*, 563-577.
45. Barickman, T.C.; Ku, K.-M.; Sams, C.E. Differing precision irrigation thresholds for kale (*Brassica oleracea* L. var. *acephala*) induces changes in physiological performance, metabolites, and yield. *Environmental and Experimental Botany* **2020**, *180*, 104253, doi:<https://doi.org/10.1016/j.envexpbot.2020.104253>.
46. Bhandari, S.R.; Lee, J.G. Ripening-dependent changes in antioxidants, color attributes, and antioxidant activity of seven tomato (*Solanum lycopersicum* L.) cultivars. *Journal of Analytical Methods in Chemistry* **2016**, 2016.