A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Transcriptomic and Metabolomic Analyses to Reveal New Cellular Responses of Harvested Strawberry Fruit Exposed to Short-term 30% CO_2

단기간 30% 이산화탄소에 노출된
딸기 과실의 세포 반응 구명을 위한
전사체 및 대사체 분석

FEBRUARY, 2018

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MAJOR IN HORTICULTURAL SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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UNDER THE DIRECTION OF DR. EUN JIN LEE SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

To improve the storability of strawberry fruit, we subjected harvested fruits to short-term exposure to 30% carbon dioxide (CO_2) and used transcriptomic and metabolomic analyses to identify new cellular responses induced by this treatment. Fruits were stored at 10°C for 10 days after 3 h exposure to 30% CO_2 (treatment) or air (control). The treatment inhibited fruit decay and softening. Transcriptomic analyses revealed that expression levels of the cell wall-degrading enzymes expansin,

pectinesterase, and β-xylosidase were significantly reduced and heat-shock proteins were significantly increased by CO_2 treatment. Metabolite profiling revealed that glucose, quinic acid, succinic acid, arabinose and glycerol were significantly increased by the treatment, which suggests that the exposure delayed fruit ripening. Transmission electron microscopy showed that disintegration of the middle lamella in the cell wall was inhibited by the treatment. Pectin content in cell walls was 46% higher in treated fruit than in control fruit at 3 days post-storage. These results confirm that short-term 30% CO_2 treatment reduces pectin decomposition in the cell wall by reducing the activity of cell wall-degrading enzymes and inducing abiotic stress responsive genes in harvested fruits.

Keyword: carbon dioxide, *Fragaria* × *ananassa*, metabolite, transcriptome, plant cell wall, postharvest

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INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is a popular horticultural crop cultivated worldwide for its unique flavour and juicy texture. It is a good source of vitamin C and other antioxidants (Cordenunsi *et al.*, 2005), but is easily perishable due to quick ripening, mechanical damage, and fungal decay after harvest. It has a short shelf life due to its rapid postharvest physiological metabolism. One day after storage at 6°C, levels of sucrose, a major sugar in strawberry fruit, reach undetectable levels due to rapid postharvest metabolism (Cordenunsi *et al.*, 2003). Although the storage period differs among strawberry cultivars, the average storage period is usually only 3–5 days (Cordenunsi *et al.*, 2003).

Researchers have studied postharvest technologies to increase strawberry storability and maintain fruit firmness. As a basic approach, low-temperature storage efficiently prolongs shelf life although it causes undesirable sensorial and nutritional changes (Cordenunsi *et al.*, 2005). Other postharvest technologies such as treatment with 1-methylcyclopropene (Jiang *et al.*, 2001), ozone (Pérez *et al.*, 1999), ultraviolet irradiation (Pombo *et al.*, 2009), or chitosan (Hernández-Muñoz *et al.* 2008) have been tested to maintain fruit freshness and hardness. Similarly, carbon dioxide (CO_2) can be applied to strawberry fruit after harvest.

CO_2 is required for photosynthesis, and plant biomass can be gained by increasing environmental CO_2 levels under sufficient water and nitrogen conditions

(Reich *et al.*, 2014). CO_2 has been used as a fertiliser in plant factories, and could be used as a postharvest treatment of horticultural crops. Controlled-atmosphere (CA) storage, a technique for reducing oxygen (O_2) concentrations and increasing CO_2 concentrations in storage chambers, is widely used to suppress the postharvest respiration metabolism of produce. For example, CA storage inhibits the expression of sucrose-, fructose-, and glucose-degrading enzymes in apples, which maintains their initial high sugar levels (Zhu *et al.*, 2013). However, CA storage should be conducted carefully, because it can cause undesirable physiological disorders (e.g., internal tissue browning) (Saquet *et al.*, 2003).

As a pre-storage technique, short-term exposure to high CO_2 levels is considered the best method for prolonging freshness, particularly for overseas export of strawberries. Several studies have experimentally demonstrated that such exposure can improve crop storability. For example, exposure to 30% CO_2 for 3 h displays delayed decay in broccoli caused by mould growth, ethylene production, and senescence (Wang, 1979). Strawberries exposed to 20% CO_2 for 12 or 48 h are firmer than fruits exposed to normal air during storage for 3 days (Ueda and Bai, 1993). CO_2 influences cell wall calcium binding, increasing fruit firmness (Wang *et al.*, 2014). Although positive effects of high CO_2 have been observed in horticultural crops, the cellular response mechanism induced by CO_2 remains completely unknown in plants.

With the development of gene, protein, and metabolite analysis technologies, omics studies have been used to understand comprehensive qualitative and quantitative plant responses under various conditions. Integrating such data would

offer a greater understanding of plant responses to external stimuli (Bino *et al.*, 2004). For example, dynamic responses of rice to *Chilo suppressalis* attack have been investigated by combining transcriptome and metabolome analyses (Liu *et al.*, 2016). Transcriptomic analyses have shown that hot water brushing upregulates abiotic and biotic stress-related genes and sugar flavonoid metabolism-related genes but downregulates chlorophyll degradation-related genes in harvested mango fruit (Luria *et al.*, 2014). Metabolic information on changes between natural versus exogenous ethylene-induced ripe kiwifruit have focused on sensory differences for consumer acceptance (Lim *et al.*, 2016; Luria *et al.*, 2014). In post-harvest, most omics studies have focused on understanding the physiology of fruit ripening and senescence.

In this study, we first elucidated the cellular responses induced by short-term 30% CO_2 in harvested strawberry fruit by using transcriptomic and metabolomic approaches. We determined the number of up- and down-regulated genes, structure of cell wall, and transcript levels of cell wall degradation enzymes and pathogenesis related genes. Our results provide new information on the plant responses induced by CO_2 as well as on the biological functions of CO_2 at the cellular level. Furthermore our results help to develop eco-friendly postharvest technology using CO_2 in horticulture industry.

MATERIALS AND METHODS

Strawberry fruit and CO_2 treatment

'Seolhyang' strawberry fruits (*Fragaria* × *ananassa* Duch.) were harvested at the 80% red-coloured stage in Nonsan, Korea. After harvest, the fruits were placed in two acrylic chambers at 25°C. In the first box, 30% CO_2 was provided for 3 h. As a control, air was provided for 3 h in the other chamber. To achieve an air composition of 30% CO_2, 100% CO_2 was supplied at a rate of 10 mL·min^{-1} for 10 min, and then 50% CO_2 was supplied at a rate of 1 mL·min^{-1} for 3 h. In the air treatment, air was supplied at a rate of 1 mL·min^{-1} for 3 h under the same conditions as the CO_2 treatment. The CO_2 concentrations of the chamber were measured every 30 min using a YL6500 gas analyser (Younglin, Anyang, Korea). After treatment with CO_2 or air for 3 h, the fruits were removed from each chamber and stored for 10 days at 10°C. The relative humidity of the storage chamber was maintained at 85~90%.

Fruit firmness and decay

Fruit firmness was determined using a CT-3 texture analyzer (Brookfield Co., Middleborough, Massachusetts, USA) with a disc probe of 100 mm, a rate of 5 mm s^{-1}, and a strain of 5 mm. The decay rate was visually confirmed during the storage period by counting the number of fungal decayed fruit.

Microbial analysis of strawberry fruit surface

Microbes on the fruit surface were cultivated in a 3M™ Petrifilm Aerobic Count Plate and 3M™ Petrifilm Yeast and Mold Count Plate (3M, USA) to test a possible sterilising effect of CO_2. Fruits washed with 0.01% sodium hypochlorite for 1 min were used as a control. The microbe plates were cultivated following the manufacturer's instructions.

RNA extraction and cDNA synthesis

Frozen fruit tissue was completely ground into a fine powder using a mortar and pestle in liquid nitrogen. Total RNA was extracted using a Ribospin™ Seed/Fruit Kit (GeneAll, Seoul, Korea) following the manufacturer's instructions. Then, 5 μL RNA extraction solution was used for the transcriptome analysis and 5 μg total RNA was used for the cDNA synthesis for qRT-PCR. cDNA was synthesised using an amfiRivert Platinum cDNA Synthesis Master Mix Kit (GenDEPOT, Baker, TX, USA).

RNA-sequencing and transcriptome analysis

For transcriptome analysis, 3 biological replicates were sampled at the 3 stages listed below. Stages for RNA-sequencing were 0D (immediately after harvest), 1D (1

day after air exposure, control), and 1DT (1 day after 30% CO_2 exposure for 3 h, treatment). RNA sequencing was performed with a HiSeq 2500 system using the 250 bp paired end at the National Instrumentation Center for Environmental Management, Seoul National University, Korea. Over 51 Gbp of raw sequences were obtained from the nine samples (three biological replicates per '0D', '1D', and '1DT', respectively), ranging from 2.8 to 7.8 Gbp per sample. The raw reads were deposited in the NCBI/EBI/DDBJ Short Read Archive (Accession number: PRJNA421027). The raw reads were trimmed and filtered by removing low-quality reads, adaptor sequences, contaminants, and duplicates. Preprocessed reads were assembled *de novo* using CLC Genomics Workbench ver. 3.7.1, Trinity ver. 2.0.2, and Velvet ver. 1.1.04 followed by Oases ver. 0.1.21 and the gene sets were obtained by merging the results (Grabherr *et al.*, 2001; Ahn *et al.*, 2015). To determine the transcript expression level, normalised values considering the transcript length and depth of coverage were calculated. DEGs were selected using a threshold of ≥2-fold up- or downregulated genes with a *p*-value < 0.05. We performed functional annotation by running a BLAST search using the NCBI non-redundant protein database (https://www.ncbi.nlm.nih.gov/). The unigenes were annotated with GO terms using Blast2GO ver. 2.6.5 (Conesa *et al.*, 2005). Functional annotations for DEGs were further performed using the KEGG pathway database and MapMan 3.6.0 (http://mapman.gabipd.org).

qRT-PCR analysis

For qRT-PCR, the cDNA solution was diluted to 50 ng·μL^{-1}. The qRT-PCR reaction was performed using 2X Real-Time PCR Master Mix including SYBER Green 1 (BioFACT^{TM}, Daejeon, Korea) and the CFX Connect^{TM} Real-Time System (BIO-RAD, Hercules, CA, USA) for 40 cycles under the following conditions: 95°C for 15 min followed by 40 cycles of 95°C for 20 s, 55°C for 40 s, and 72°C for 20 s. Primer pairs were designed manually based on the *de novo* assembly reference gene set and are listed in **Table 1**. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001) and normalised using the expression levels of the housekeeping gene *GAPDH* (AB363963.1). Three biological replicates were included for each qRT-PCR reaction.

Analysis of polar metabolites by GC-MS

Metabolites were extracted according to a previously described method (Lisec *et al.*, 2006) with some modifications. First, 50 mg freeze-dried strawberry fruit powder was vortexed with 1.2 mL methanol. Then, 1 μL of the sample was injected into the gas chromatography-mass spectrometry (GC-MS) ISQ^{TM} LT system (Thermo Fisher Scientific, Waltham, MA, USA) using an auto sampler. Three biological replicates were included for each analysis. The metabolite data were normalised and scaled, and were used for dendrogram construction, heatmap cluster analysis, and partial least squares-discriminant analysis (PLS-DA) using MetaboAnalyst 3.0 software (www.metaboanalyst.ca). Auto-scaling and sum-based normalisation procedures were performed. The metabolic pathway was illustrated based on the MetaboAnalyst 3.0 pathway analysis.

Table 1. Primer information used for qRT-PCR.

Genes	Primer sequence	Product size
Expansin	F: CCTACGTGGGCGTCTAT	170
	R: AACCTTCAAACATACTAGCAAA	
Pectinesterase	F: ACAGAAGCTCAGGCTTTCA	129
	R: CTCACTCGATAACACAGCTTA	
β-Xylosidase	F: TCTCTTAGCCAAAGTGACCA	160
	R: AGCTAACCAAAACAGAGCAG	
Chaperonin 60 subunit α 2	F: TAGTTGCTGACCCTTCTACC	140
	R: TACAGCAATACCACCACAGA	
17.9 kDa class II heat shock protein	F: CCAAAGAAGCCCAAGACTA	129
	R: CATATGGAAAGCACACTGGT	
Heat shock cognate 70 kDa protein 2	F: GCTCCTGACATGGGCAT	148
	R: CTGGACCTCTCCCCGATA	
Heat shock cognate 70 kDa protein	F: TTAAGCTGAGAATGTGCAGG	143
	R: CTACCATAGCTTTTGAGTTCCA	
Pathogenesis-related protein 10	F: AGCCTCCTCCACAATTCATT	133
	R: GACGAAAGCCTTGTACAATTTG	
Pathogenesis-related homeodomain	F: AGGTAAAACGAAGGAGAAGC	154
	R: CTTTGCCTTGGCTATTTGAA	
Dehydroascorbate reductase	F: CTTACAAGCTCCATCTCATCA	135
	R: CAAGAATCCCGACAAGCAC	
Glutatione-S-transferase 1	F: AAGCACTCTTTTGTGCCTC	134
	R: ACGATGAATTCCACTTGAAATAC	
Glutatione-S-transferase 2	F: TATTCAAATGCTCAGTCGGC	145
	R: AAGTCAGCAACAATGTCCAC	
Peroxidase	F: GTCGCTGTGATTAGGACATG	124
	R: TTCGCATAGAACCCTACTTT	
Aquaporin TIP4-1	F: TGCTCTTGATTTGCAGAGAC	141
	R: TGACAAACTCGACGATAAGC	
Aquaporin PIP1-3	F: AAGGAAGAGGATGTGAGGTT	155
	R: ATTCCAGCTCTCCAGAAAGA	
GAPDH	F: CCTGCTCTCAATGGCAAATT	155
	R: GATCTCTTCATCTTTCCCTCA	

F, forward; R, reverse.

TEM image

Fresh fruits were used for transmission electron microscopy (TEM). The shoulder part of the fruit was cut into a regular triangle with a length of 1 mm, and tissue was cut into slices 1~2 mm thick. Primary fixation was conducted using Karnovsky's fixative (EMS, Hatfield, PA, USA) for 2 h. The solution was exchanged for 0.05 M sodium cacodylate buffer, and the buffer was replaced every 10 min three times for washing. The solution was exchanged for 2 mL of 0.1 M cacodylate buffer : 2% osmium tetroxide (1:1, v/v), and the sample was maintained for post-fixation. After 2 h, the sample was rinsed twice with distilled water. For en bloc staining, the sample was submerged overnight in 0.5% uranyl acetate. To dehydrate the samples, the solution was replaced sequentially with 30%, 50%, 70%, 80%, and 90% ethanol (v/v) in 10 min intervals. During the last step of dehydration, solution was replaced with 100% ethanol three times in 10 min intervals. The sample was dipped in propylene oxide for 10 min twice for transition. The solution was sequentially replaced with 2:1 and 1:1 (v/v) propylene oxide and Low Viscosity Embedding Media Spurr's Kit Resin (EMS, Hatfield, PA, USA) mixtures in 1 h intervals for infiltration. The sample was submerged in Spurr's resin overnight in a desiccator, and then placed in a mould and polymerised at 70°C for 24 h using fresh Spurr's resin. An EM UC7 ultramicrotome (Leica, Wetzlar, Germany) was used for sectioning. The sample was examined and photographed using a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan).

Cell wall extraction and polyuronide assay

Cell wall extraction was conducted according to previously described methods (Chatkaew and Kim, 2013) with some modifications. Cell wall extract was used for the polyuronide assay. First, 10 mg cell wall extract was placed in a glass test tube and 2 mL cold sulphuric acid was added. After waiting 2 h, 23 mL distilled water was added. Then, 200 μL diluted sample was transferred to a new glass test tube. Following the methods of Gross and Wang (1984) with some modifications, this solution was used to measure polyuronide content. Next, 20 μL potassium sulphamate/sulphamic acid solution (pH 1.6) and 1.2 mL sulphuric acid containing 75 mM sodium tetraborate were added to 200 μL of the sample and vortexed. The mixture was heated at 100°C for 20 min. After cooling on ice, 40 μL 0.15% (v/v) *m*-phenyphenol in 0.5% NaOH was added and maintained for 20 min in 25°C. After vortexing, 200 μL aliquots of extract were transferred to the wells of a 96-well plate and the absorbance was detected at 525 nm using a Multiple Plate Reader (Perkin Elmer, Waltham, MA, USA).

Statistical analysis

The experiments followed a random design with three biological replicates. The data are the means ± standard deviations of three replicates. The independent two-sample *t*-test and one-way ANOVA were conducted using SPSS ver. 22.0 (IBM, Armonk, NY, USA).

RESULTS

Short-term 30% CO_2 inhibits fruit decay

Fruits treated with 30% CO_2 had less grey mould than those treated with air (**Fig. 1A**). Fruit decay began 5 days after storage, and CO_2 treatment reduced decay. At 10 days post-treatment, 27% of control fruits had rotted, compared to only 19% of CO_2-treated fruits (**Fig. 1B**). To see whether CO_2 could have a sterilising effect, we counted the number of microbes on the fruit surface, which did not significantly differ between the groups (**Fig. 2**). This confirmed that the reduced decay and improved storability by CO_2 exposure were not directly due to a sterilising effect but rather to biological responses in harvested fruits induced by the treatment.

CO_2 treatment also maintained fruit firmness (**Fig. 1C**), which was 10.25 N at the harvest stage; it gradually decreased with storage duration to 7.86 N and 9.16 N in air- and CO_2-treated strawberry fruits, respectively, after 10 days.

(A) Air (control)

30% CO_2 for 3 h

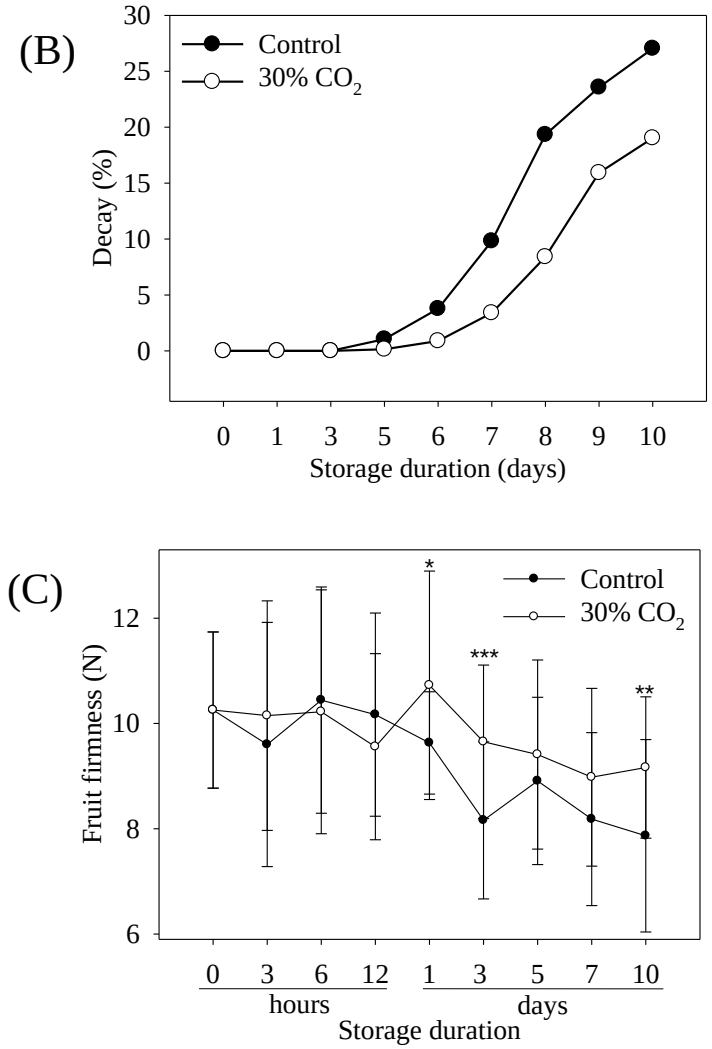


Fig. 1. Effect of short-term 30% CO_2 on strawberry fruit quality during storage. Appearances of strawberry fruit exposed to air (up) and 30% CO_2 for 3 h (down) later 10 days after storage (A), fruit decay (B), fruit firmness (C) during storage, respectively. Data are means of replicates ± standard deviations (n=30 for each replicate). *, **, and *** represent significant differences at $P < 0.05$, 0.01 and 0.001, respectively.

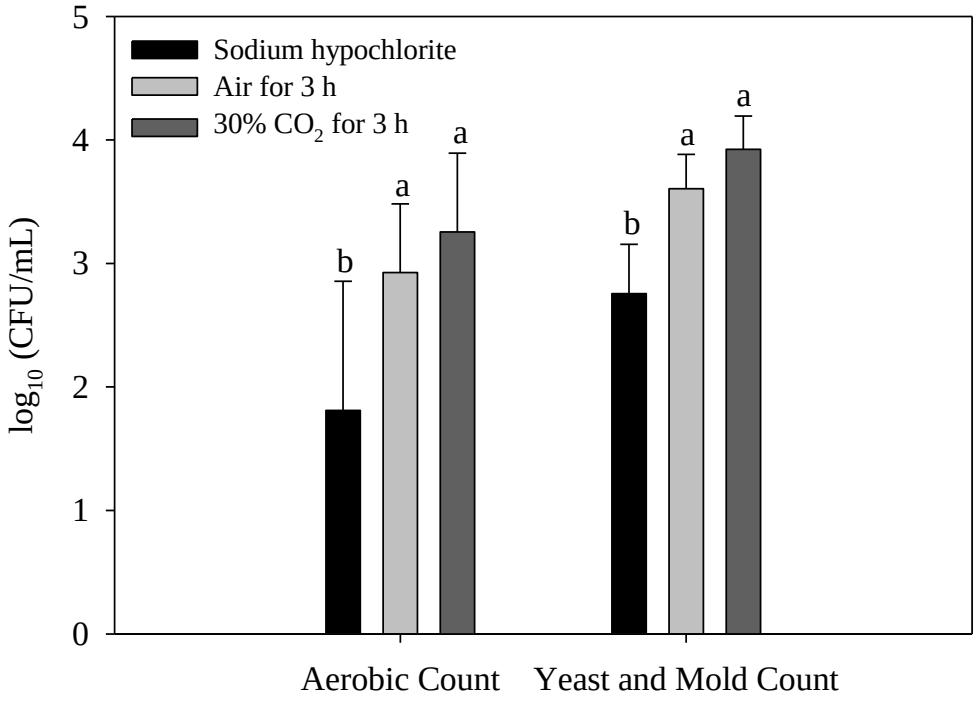


Fig. 2. Microbial analysis using 3M petrifilm plate. Data are means ± standard deviations of 5 replicates. Duncan's multiple test were conducted to each aerobic count and yeast and mold count. (P<0.05). CFU, colony forming unit.

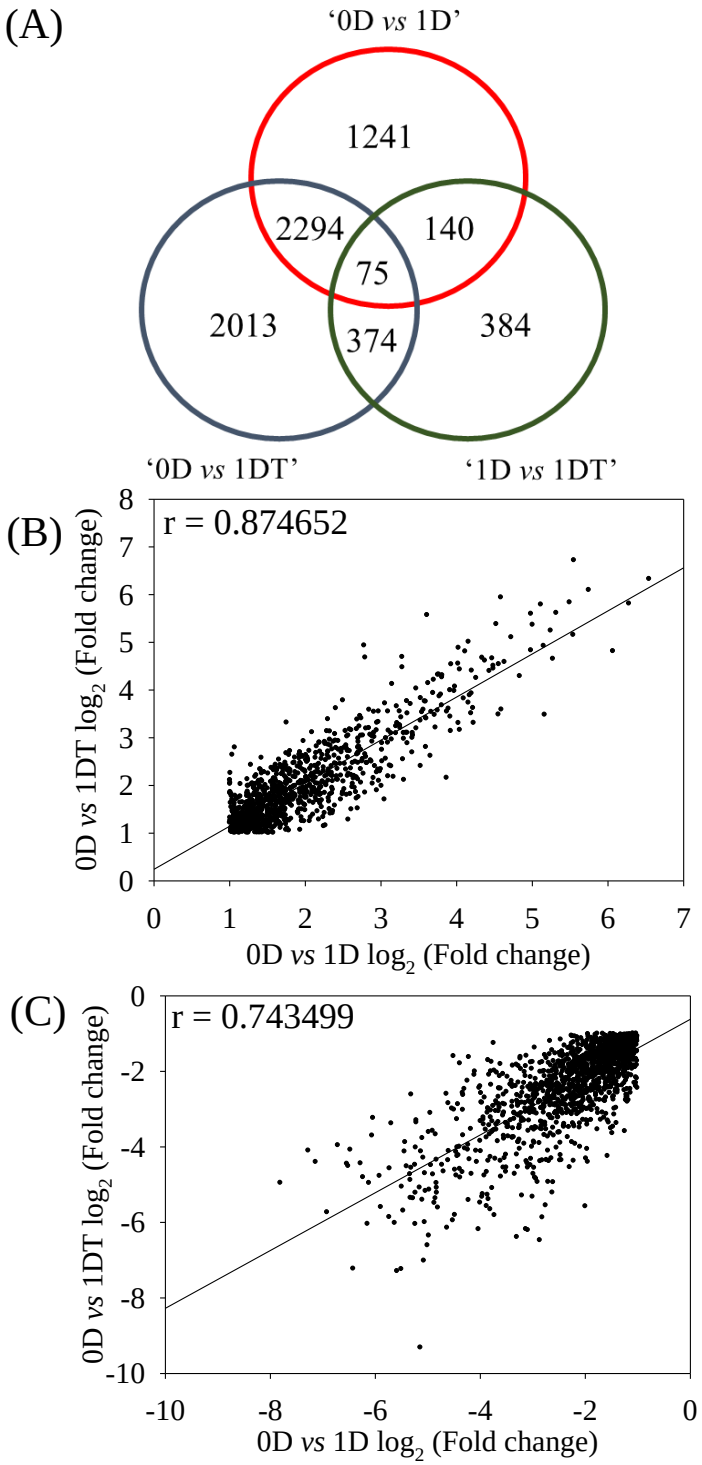
Short-term 30% CO_2 DEGs were enriched on pathways related to cell wall, sucrose, and secondary metabolism

A total of 63,715 contigs with 70.7 Mb were assembled *de novo*. The average contig length was 1,110 bp, and the N50 was 1,449 bp (**Table 2**). Most contigs (96%) were annotated using Gene Ontology (GO). Three biological replicates for each group at '0D' (immediately post-harvest), '1D' (1 day after air treatment for 3 h; control), and '1DT' (1 day after 30% CO_2 treatment for 3 h; treatment) were pairwise compared (**Fig. 3A**). In the case of the '0D vs. 1D' pair, 3,750 contigs were differentially expressed after 1 day of storage at 10°C. The transcriptome changes were conserved by sharing 2,369 differentially expressed genes (DEGs) between the treatment and control, which accounted for 50% of DEGs from '0D vs. 1DT' and 63% of DEGs from '0D vs. 1D'. Furthermore, 2,369 shared DEGs showed consistent expression changes between '0D vs. 1D' and '0D vs. 1DT', with Pearson's correlation coefficients of 0.8746 and 0.7434 for up- and downregulated DEGs, respectively (**Figs. 3B and C**). The remaining DEGs detected from the comparison between '1D' and '1DT' were used to investigate the effects of 30% CO_2.

The transcriptome gene expression profile were validated by quantitative real-time (qRT) PCR. The expression levels of 15 DEGs in '1D vs. 1DT' showed consistent expression changes in the qRT-PCR results, with a Pearson's correlation coefficient of 0.8256 (**Fig. 3D**).

Table 2. *De novo* assembly statistics.

Analytical catagory	
Total length (bp)	70,742,896
Number of contigs	63,715
Average length (bp)	1,110.30
Median length (bp)	839
Maximum length (bp)	25,685
Minimum length (bp)	297
N50 length (bp)	1,449
N80 length (bp)	751
GC content (%)	44


(A)
'0D vs 1D'
1241
2294
140
75
2013
374
384
'0D vs 1DT'
'1D vs 1DT'
(B)
r = 0.874652
0D vs 1DT log2 (Fold change)
0D vs 1D log2 (Fold change)
(C)
r = 0.743499
0D vs 1DT log2 (Fold change)
0D vs 1D log2 (Fold change)

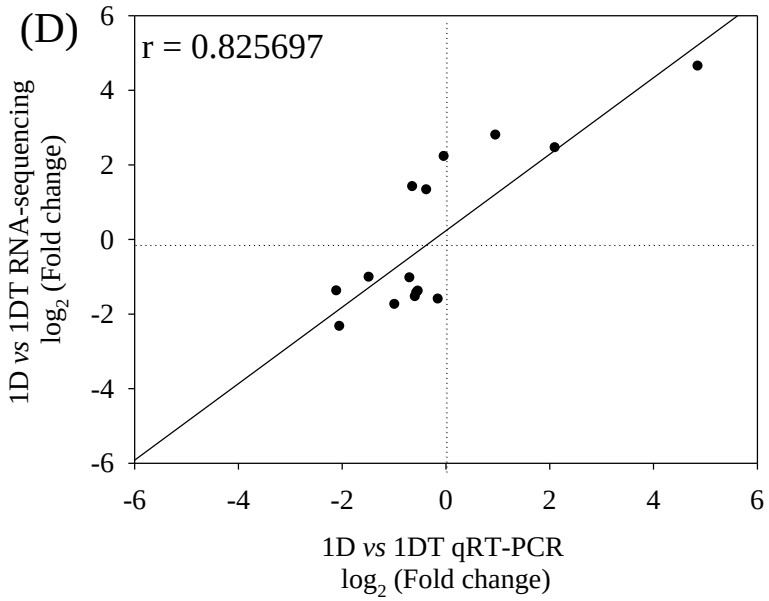


Fig. 3. Number of DEG and DEG correlation. A Venn diagram of DEG between samples of '0D *vs* 1D', '0D *vs* 1DT', and '1D *vs* 1DT' are respectively shown in (A). Pearson's correlation efficient of 958 shared up-regulated DEGs in '0D *vs* 1D' and '0D *vs* 1DT' is shown in (B). Pearson's correlation efficient of 1,409 shared up-regulated DEGs in '0D *vs* 1D' and '0D *vs* 1DT' shown in (C). Pearson's correlation efficient of expression levels between RNA-sequencing and qRT-PCR shown in **Fig. 5** in '1D *vs* 1DT' is also shown in (D).Three biological replicates were used for all analysis.

KEGG (**Table 3**) and MapMan (**Fig. 4**) were used to review the DEG characteristics. Using the '0D vs. 1D' DEGs, physiological changes after 1 day of storage were observed. Genes responding to cell wall, sucrose, lipids, and secondary metabolism showed changes in the MapMan pathway (**Fig. 4A**). These pathways were represented and specified from the enriched KEGG pathways, including 'Starch and sucrose metabolism', 'Flavonoid biosynthesis', and 'Glycerolipid metabolism' (**Table 3**). The MapMan profile of the '0D vs. 1DT' DEGs showed similar patterns, which suggests that the '1D' and '1DT' samples underwent similar physiological processes (**Fig. 4B**).

The enriched KEGG pathways showed differences between '1D' and '1DT', indicative of the effects of 30% CO_2 exposure, where 'Pyrimidine metabolism' was enriched in the upregulated DEGs and 'Glycosphingolipid biosynthesis' was enriched in the downregulated DEGs with the highest significance based on Fisher's exact tests (**Table 3**). Most contigs related to 'Pyrimidine metabolism' and 'Monobactam biosynthesis' were also related to amino acid biosynthesis, showing an acceleration of amino acid metabolism. Furthermore, 'Glycosphingolipid biosynthesis' and other enriched pathways of downregulated DEGs showed that cell wall-related metabolism was highly affected by CO_2. Finally, the metabolisms of fructose, mannose, galactose, and several amino acids were affected by CO_2 (**Fig. 4C, Fig. 8,** and **Table 3**).

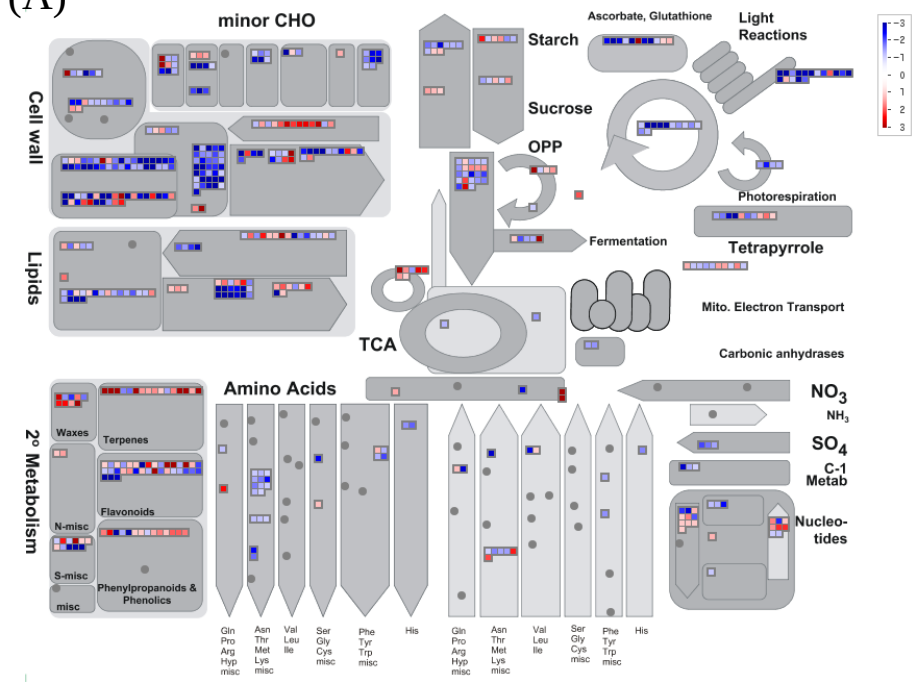
Table 3. KEGG enrichment of DEGs

		KEGG	No. of DEGs	No. of total genes	p value
0D vs 1D	up	Starch and sucrose metabolism	69	563	3.10E-07
		Pentose and glucuronate interconversions	36	235	2.37E-06
		Metabolism of xenobiotics by cytochrome P450	16	119	0.00490
		Retinol metabolism	10	57	0.00462
	down	Methane metabolism	86	226	2.20E-16
		Mannose type O-glycan biosynthesis	82	160	2.20E-16
		Carbon fixation pathways in prokaryotes	48	203	2.20E-16
		Flavonoid biosynthesis	22	81	3.37E-11
		Metabolism of xenobiotics by cytochrome P450	20	119	2.99E-07
		Steroid biosynthesis	7	17	2.16E-05
		Carbon fixation in photosynthetic organisms	24	247	0.00014
		Glycerolipid metabolism	15	154	0.00199
		Pyruvate metabolism	26	351	0.00373

Table 3. KEGG enrichment of DEGs (continued)

		KEGG	No. of DEGs	No. of total genes	p value
0D vs 1DT	up	Lysine biosynthesis	41	208	2.20E-16
		Fatty acid biosynthesis	39	134	2.20E-16
		Monobactam biosynthesis	24	33	2.20E-16
		Methane metabolism	13	226	3.03E-05
	down	Amino sugar and nucleotide sugar metabolism	27	253	2.20E-16
		Ether lipid metabolism	15	79	7.19E-15
		Glycosphingolipid biosynthesis - ganglio series	6	48	9.65E-06
		Glutathione metabolism	8	196	0.00059
		Other glycan degradation	6	112	0.00076
		Glycosphingolipid biosynthesis - globo and isoglobo series	3	22	0.00149
		Phosphatidylinositol signaling system	5	110	0.00413
		Styrene degradation	2	9	0.00433
1D vs 1DT	up	Pyrimidine metabolism	18	417	3.85E-05
		Monobactam biosynthesis	5	33	0.00017
		Alanine, aspartate and glutamate metabolism	8	147	0.00144
		Fructose and mannose metabolism	8	165	0.00287
	down	Glycosphingolipid biosynthesis - ganglio series	5	48	0.00012
		Glycosaminoglycan degradation	5	48	0.00012
		Flavonoid biosynthesis	6	81	0.00015
		Galactose metabolism	8	184	0.00040
		Other glycan degradation	6	112	0.00076
		Sphingolipid metabolism	5	88	0.00164

(A)


minor CHO
Cell wall
Lipids
2º Metabolism
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
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OPP
Fermentation
TCA
Ascorbate, Glutathione
Light Reactions
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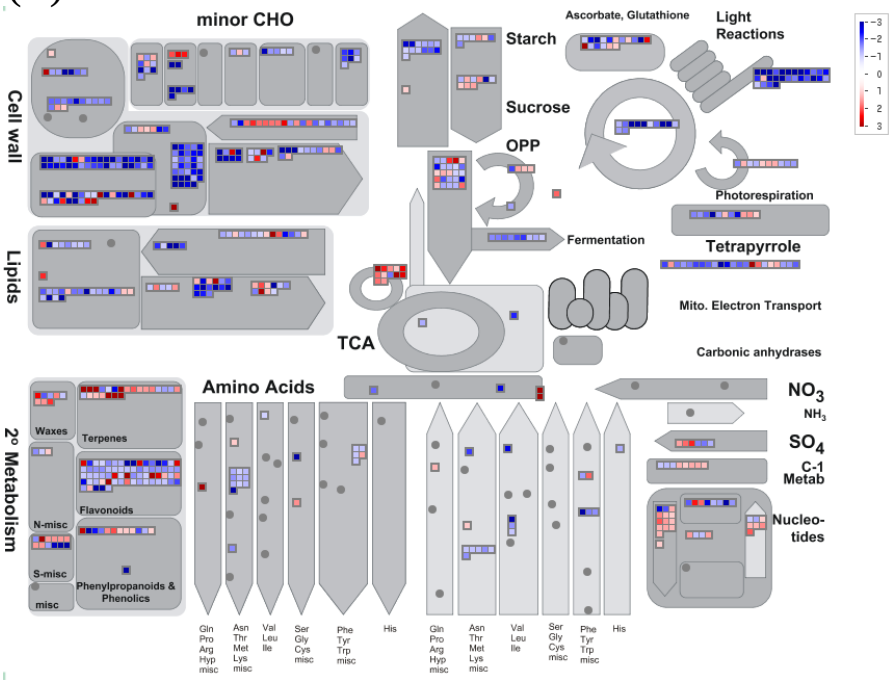
C-1 Metab
Nucleo-tides

(B)


minor CHO
Cell wall
Lipids
2º Metabolism
Waxes
Terpenes
Flavonoids
N-misc
S-misc
misc
Phenylpropanoids & Phenolics
Starch
Sucrose
OPP
Fermentation
TCA
Ascorbate, Glutathione
Light Reactions
Photorespiration
Tetrapyrrole
Mito. Electron Transport
Carbonic anhydrases
Amino Acids
Gln Pro Arg Hyp misc
Asn Thr Met Lys misc
Val Leu Ile
Ser Gly Cys misc
Phe Tyr Trp misc
His
NO3
NH3
SO4
C-1 Metab
Nucleo-tides

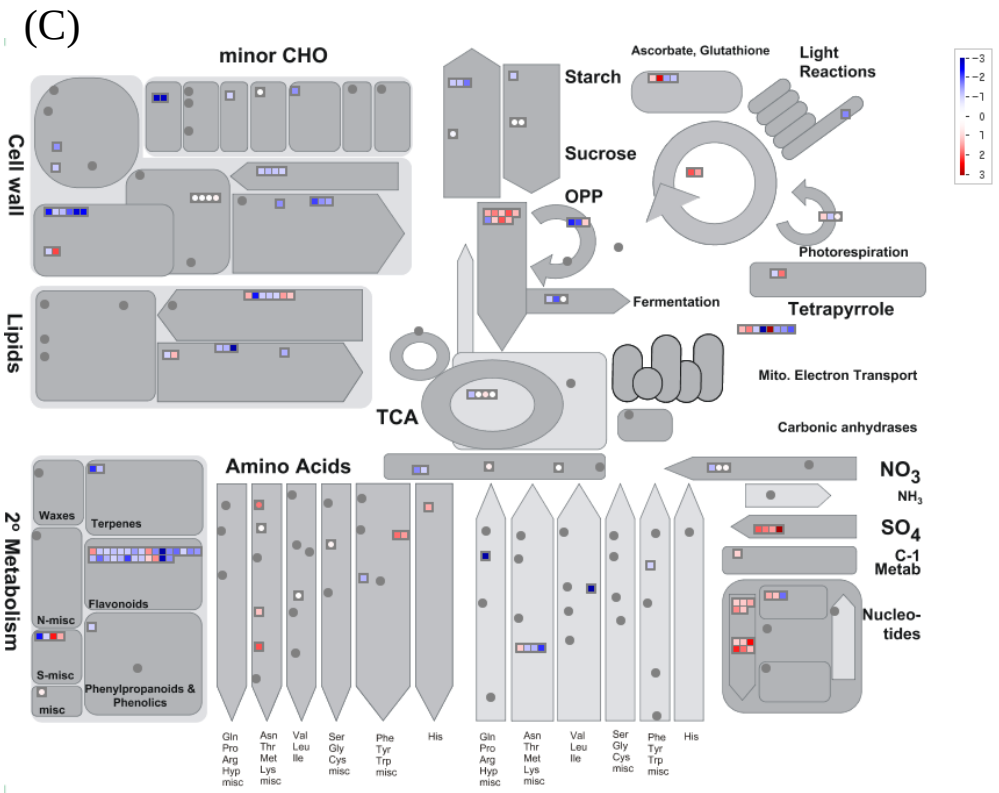


Fig. 4. Metabolism Overview by MapMan software. MapMan metabolism Overview of 0D *vs* 1D DEGs (A), 0D *vs* 1DT DEGs (B), and 1D *vs* 1DT DEGs (C). Metabolism overview pathway was showed using fold change of DEG related to overview metabolism. Log_2 (fold change) was used for expression.

Short-term 30% CO_2 inhibited the expression of cell wall degradation related genes and induced heat shock protein related genes

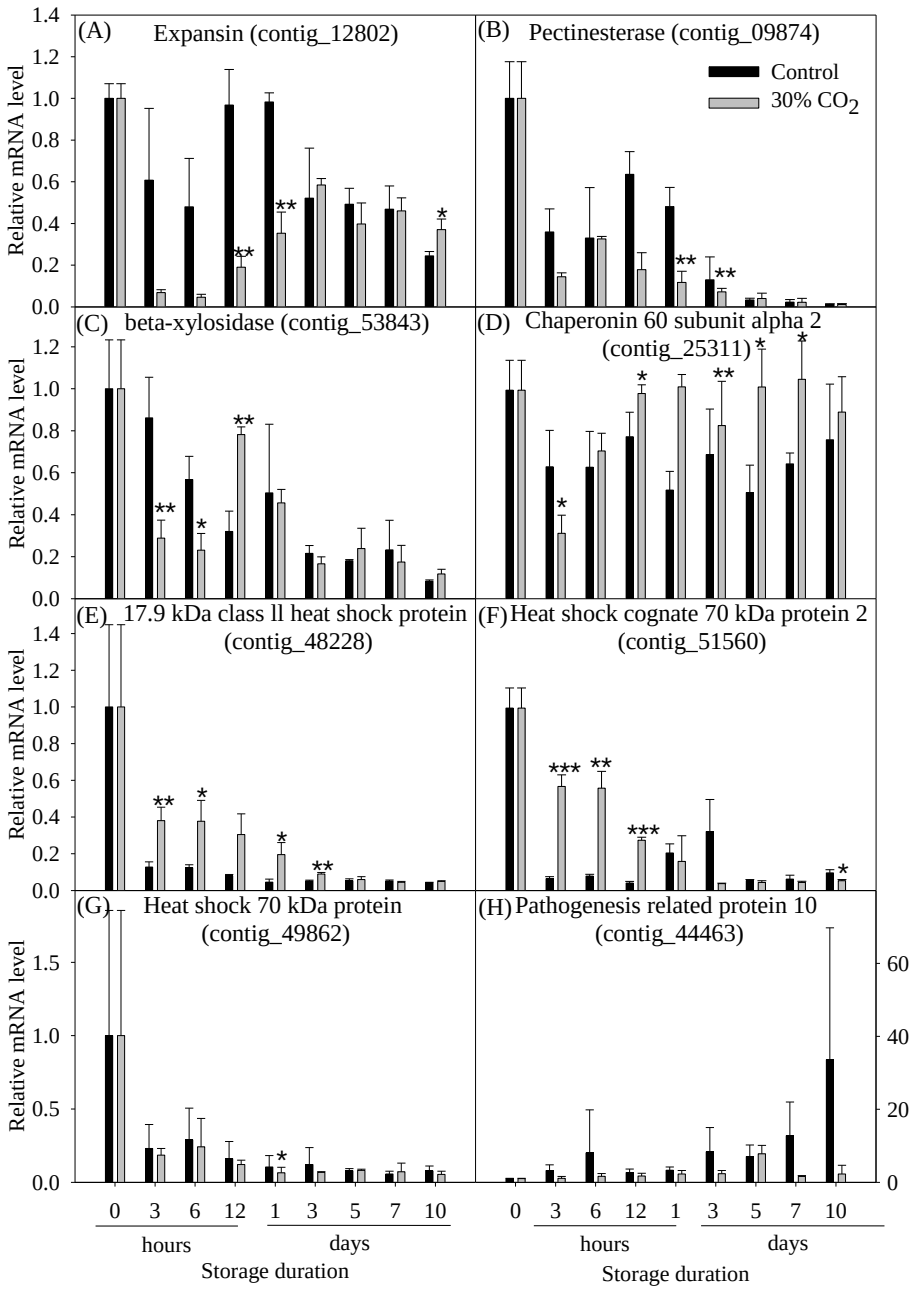
Genes related to storability were selected from the DEGs and gene expression during storage were precisely profiled (**Fig. 5**). Expansin (AF159563.1), pectinesterase (AY357183.1), and β-xylosidase (XM004295056.2) were related to cell wall degradation, and were significantly downregulated by 30% CO_2 (**Figs. 5A-C**). There are six known expansin genes in strawberry fruit, which extend the cell wall (Harrison *et al.*, 2001).

After CO_2 exposure, heat shock proteins (HSPs) of various sizes (e.g., 60 kDa [XM004295442.2], 17.9 kDa [XM004303435.2], and 70 kDa [XM004307479.2, XM004287097.2]) were significantly upregulated (**Figs. 5D-G**). Pathogenesis-related proteins (XM004296840.2, XM011464070.1), dehydroascorbate reductase (XM004307358.2), glutathione-S-transferase (XM004294173.2, XM004302642.2), and peroxidase (JX290513.1) are related to plant defence (Hammond-Kosack and Jones, 1996; Hayes and McLellan, 1999). However CO_2 exposure did not significantly impact their expressions (**Figs. 5H-M**).

Aquaporins (XM004291138.2, XM004298758.2) are membrane water channels that determine the water permeability of cells according to their activities (Chaumont *et al.*, 2005). Glissant *et al*. (2008) revealed that the expressions of various aquaporin genes have upregulated expression during postharvest ripening in grapes. In this study,

aquaporin TIP 4-1 and PIP 1-3 were downregulated 12 h after CO_2 treatment, prolonging fruit freshness (**Figs. 5N and O**).

CO_2-treated strawberry fruits showed enhanced resistance to grey mould (**Fig. 1A, up**). To confirm the effects of CO_2 on pathogen resistance in strawberry fruit, we mapped the DEG contigs in MapMan and investigated the overview pathway related to biotic stress (**Fig. 6**). Four DEGs linked to signalling and defense responses of pathogen and pest attacks were slightly induced by CO_2. However, DEGs directly involved in abiotic stress and indirectly involved in biotic stress such as hormone signalling, redox state, and cell wall metabolism were clearly induced by CO_2.


(A) Expansin (contig_12802)
(B) Pectinesterase (contig_09874)
Control
30% CO2
(C) beta-xylosidase (contig_53843)
(D) Chaperonin 60 subunit alpha 2 (contig_25311)
(E) 17.9 kDa class ll heat shock protein (contig_48228)
(F) Heat shock cognate 70 kDa protein 2 (contig_51560)
(G) Heat shock 70 kDa protein (contig_49862)
(H) Pathogenesis related protein 10 (contig_44463)
Relative mRNA level
0 3 6 12 1 3 5 7 10
hours
days
Storage duration
0 3 6 12 1 3 5 7 10
hours
days
Storage duration

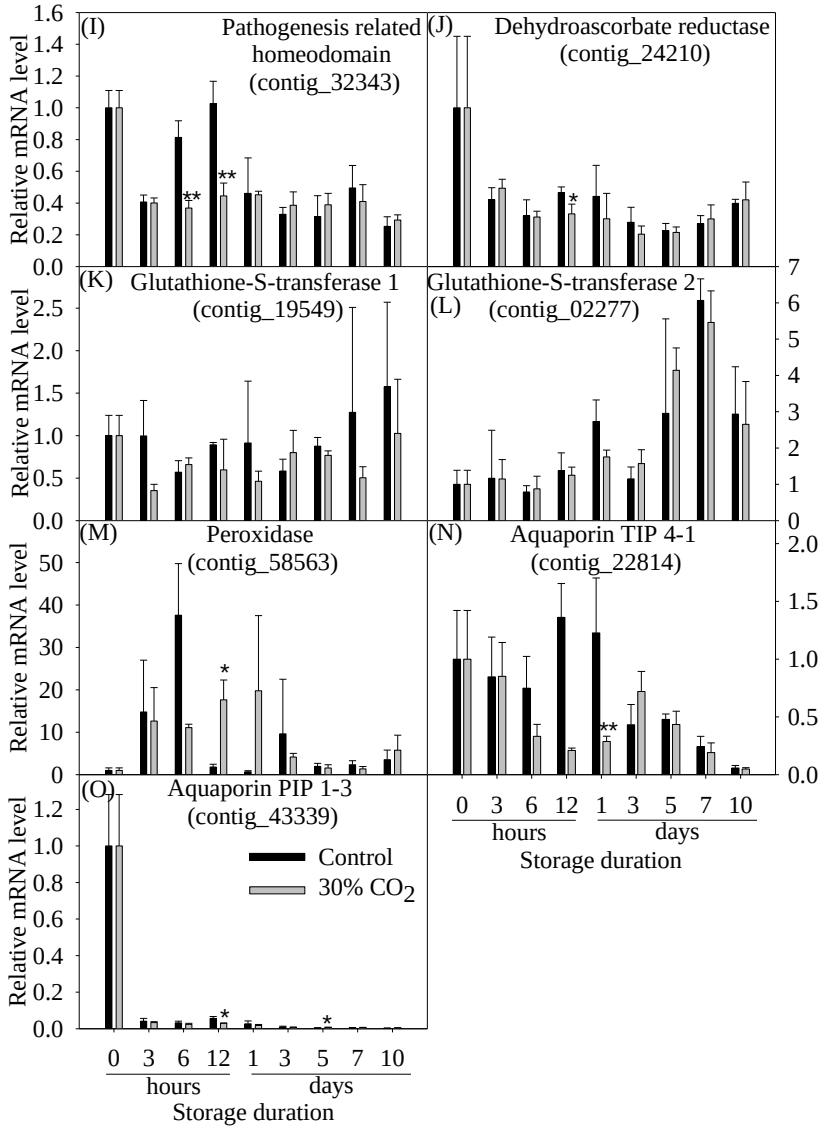


Fig. 5. qRT- PCR validation of contigs acquired by RNA-sequencing. Fruits were stored at 10°C for 10 days after exposure to air or 30% CO_2 for 3 h, respectively. Name of each gene is indicated on the top of the graph. Data are means of 3 biological replicates ± standard deviations (n=3 for each replicate). The independent two-sample t-test between air and CO_2 treatment was conducted at the same time. *, **, and *** represent significant differences at $p < 0.05$, 0.01, and 0.001, respectively.

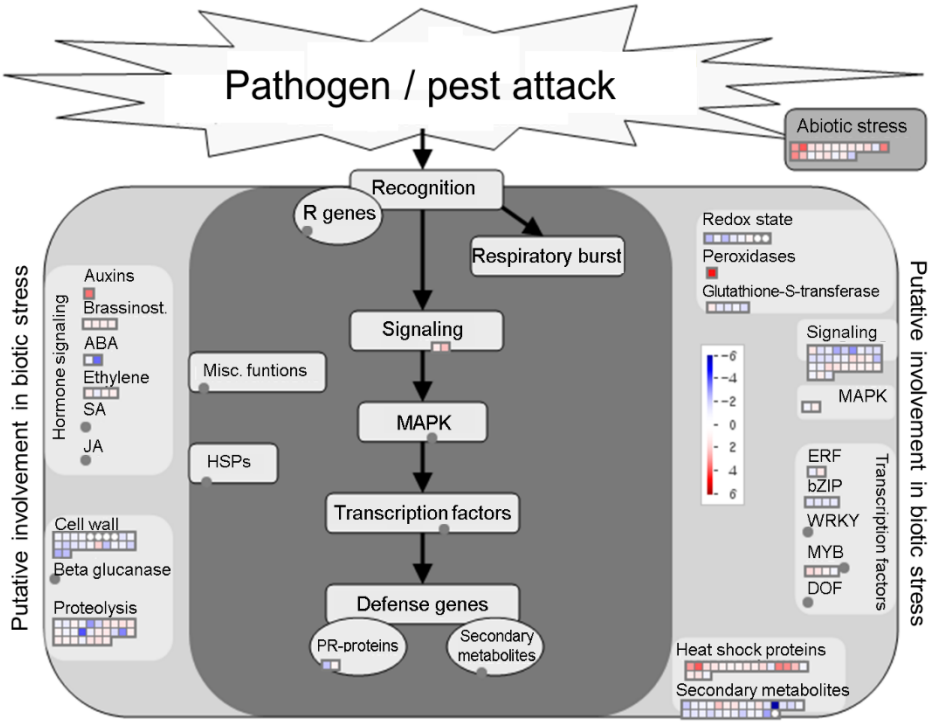


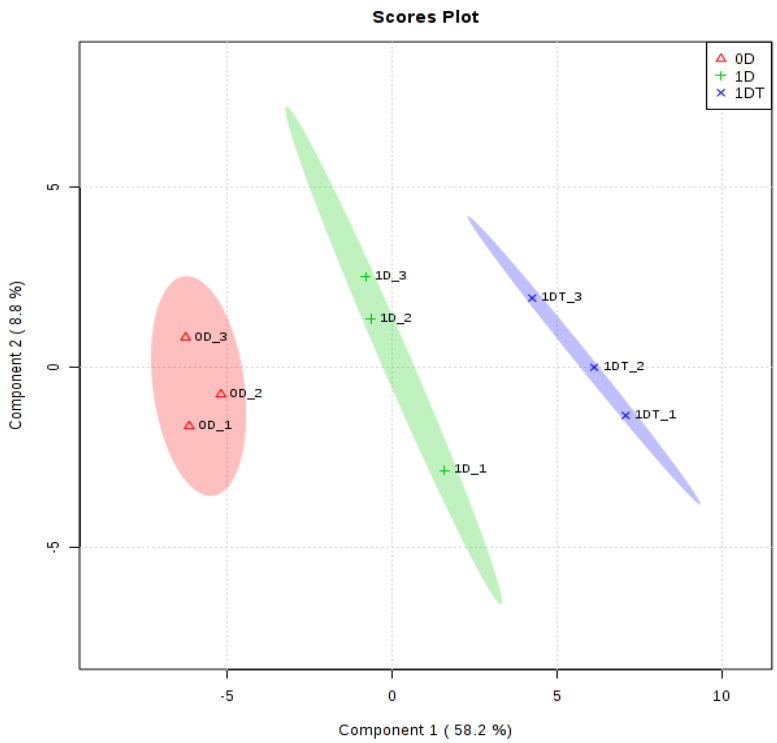
Fig. 6. Overview pathway of 1D *vs* 1DT DEG related to biotic stress by MapMan software. Overview pathway was showed using fold change of DEG related to biotic stress. Log_2 (fold change) was used for expression.

Short-term 30% CO_2 altered sucrose metabolism

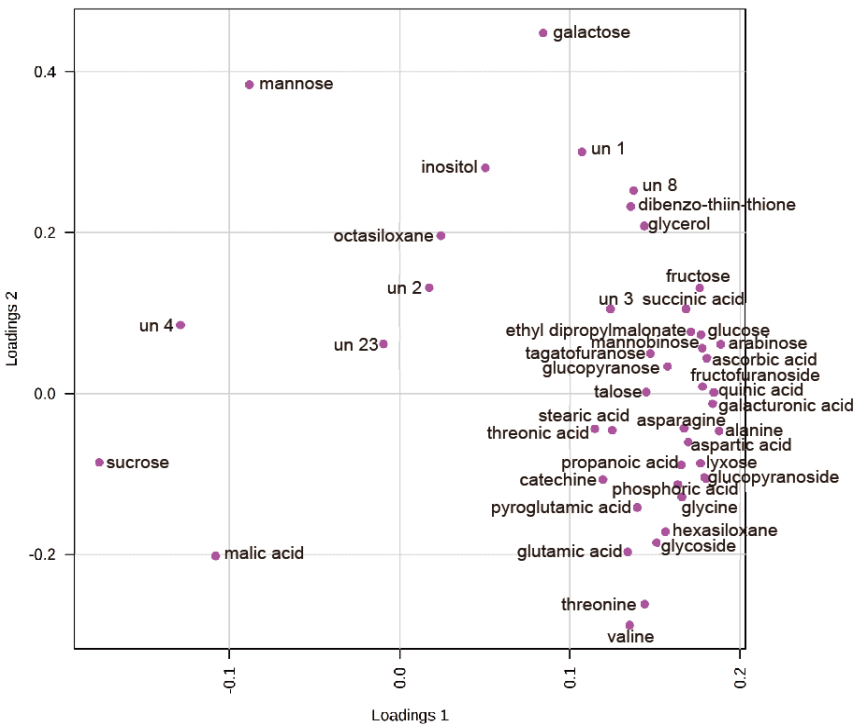
We detected and quantified 40 metabolites by GC-MS. To confirm the relationships among the metabolite changes induced by CO_2, PLS-DA was performed (**Fig. 7**). The first and second principal components accounted for 67% of the variance in the dataset, but the sample separation was the highest for the first principal component (PC1), which accounted for 58.2% of the variance. Because the groups were separated based on PC1, metabolites contributed the most to the group separation. During storage at 10°C, all fruits in the '0D', '1D', and '1DT' groups showed changes in their metabolite profiles and were separated clearly according to group (**Fig. 7A**). This is consistent with the results shown in **Figs. 1–6**, which indicates that the metabolic changes were dependent on CO_2 exposure.

Fig. 7B presents the loaded metabolites for the PLS-DA and **Fig. 7C** presents the variable importance in projection (VIP) scores of each metabolite. Concentrations of ascorbic acid and phosphoric acid increased predominantly in the 30% CO_2 treated fruits, and both compounds mainly contributed to separating the groups. Contents of saccharides such as galactose, mannobiose, glucose, and fructose which can be components of cell wall were also increased by CO_2 exposure.

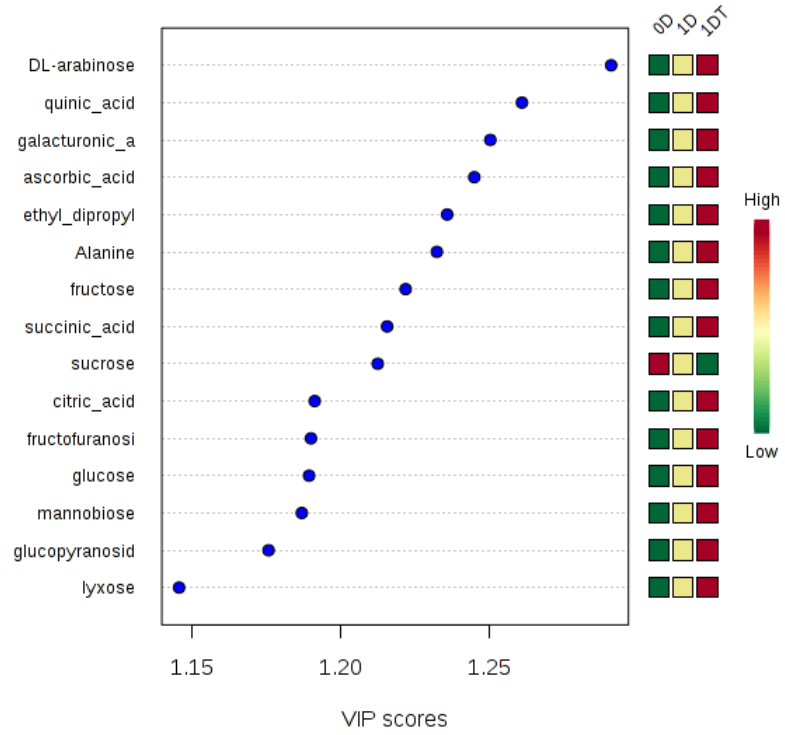
(A)


Scores Plot
0D
1D
1DT
0D_3
0D_2
0D_1
1D_3
1D_2
1D_1
1DT_3
1DT_2
1DT_1
Component 2 (8.8 %)
Component 1 (58.2 %)
5
0
-5
-5
0
5
10

(B)


galactose
mannose
un 1
inositol
un 8
dibenzo-thiin-thione
glycerol
octasiloxane
fructose
un 2
un 3
succinic acid
un 4
ethyl dipropylmalonate
glucose
mannobinose
arabinose
un 23
tagatofuranose
ascorbic acid
glucopyranose
fructofuranoside
talose
quinic acid
galacturonic acid
stearic acid
asparagine
threonic acid
alanine
aspartic acid
sucrose
propanoic acid
lyxose
catechine
glucopyranoside
phosphoric acid
pyroglutamic acid
glycine
hexasiloxane
malic acid
glutamic acid
glycoside
threonine
valine
Loadings 2
Loadings 1
0.4
0.2
0.0
-0.2
-0.1
0.0
0.1
0.2

(C)


0D
1D
1DT
DL-arabinose
quinic_acid
galacturonic_a
ascorbic_acid
ethyl_dipropyl
Alanine
fructose
succinic_acid
sucrose
citric_acid
fructofuranosi
glucose
mannobiose
glucopyranosid
lyxose
High
Low
1.15
1.20
1.25
VIP scores

(D)

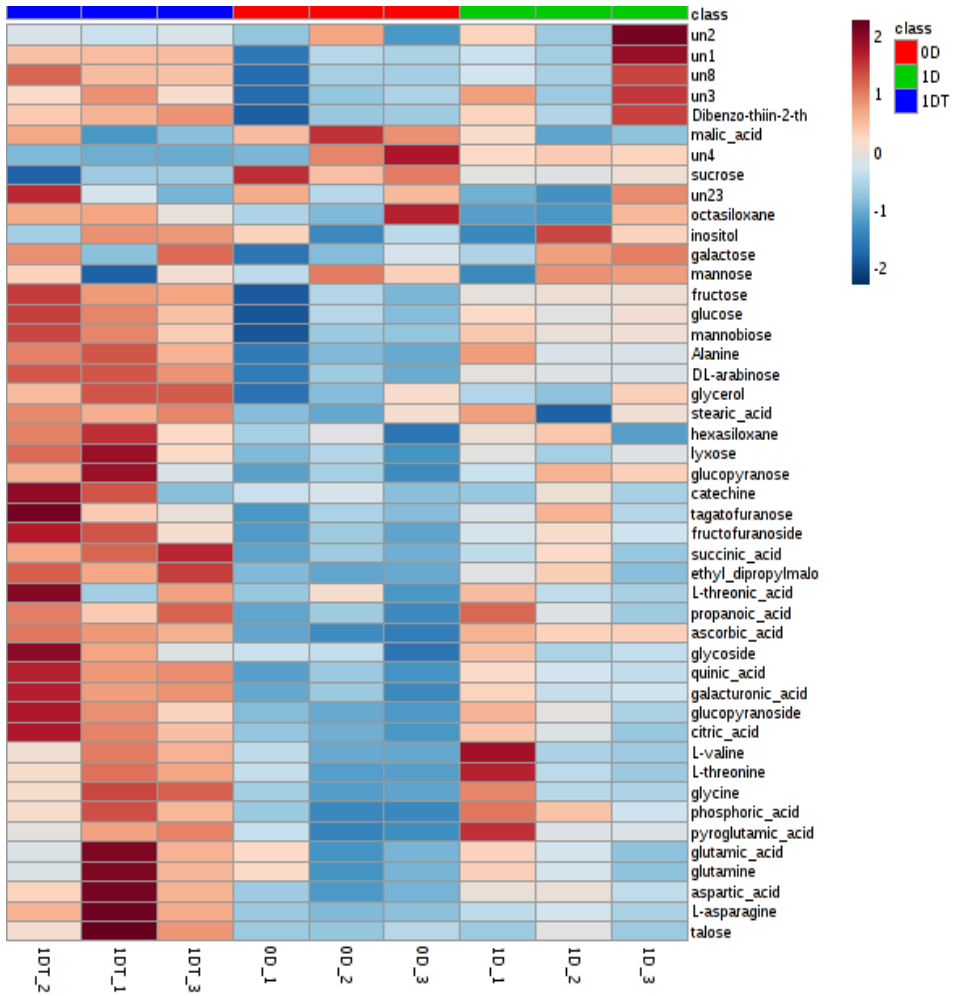


Fig. 7. PLS-DA analysis and loading plot of GC-MS based polar metabolites. Fruits were stored at 10°C for 10 days after exposure to air (control) or 30% CO_2 for 3 h, respectively. PLS-DA (A), loading plot (B), VIP score list (C), and heatmap (D) were obtained using MetaboAnalyst 3.0 software. Three biological replicates (*n*=3 for each replicate) were used for the analysis. Abbreviations are 0D (fruit at immediately after harvest), 1D (fruit at 1 day after air exposure, control), and 1DT (fruit at 1 day after 30 CO_2 exposure for 3 h, treatment), respectively.

The relative concentration of each metabolite is shown as a heatmap (**Fig. 7D**). Mapping metabolites to the general metabolic pathways based on KEGG illustrated the metabolic pathways related to the treatment (**Fig. 8**). Twenty-one metabolites (inositol, valine, fructose, glycerol, lyxose, ascorbic acid, stearic acid, malic acid, citric acid, aspartic acid, threonine, asparagine, glycine, alanine, threonic acid, glutamine, and glutamic acid, glucose, quinic acid, succinic acid, and arabinose) increased in the '1DT' samples, with significant increases in four metabolites (glucose, quinic acid, succinic acid, arabinose, and glycerol).

Candidate DEGs related to metabolite changes were listed according to their KEGG pathways (**Fig. 8**). The reduction in sucrose and increases in glucose and fructose were the greatest changes observed in the metabolomic analysis. These changes partially explained the changes in the expression of invertase inhibitor.

Downregulation of ascorbate dehydrogenase is related to increased ascorbic acid content. Many amino acid biosynthesis pathway genes were differentially expressed and showed changes in amino acid levels (e.g., glutamine, alanine, glycine, and so forth). Among the significantly changed metabolites, arabinose, a precursor of cell wall components, and quinic acid, a derivative of shikimic acid, were notable (**Fig. 8**).

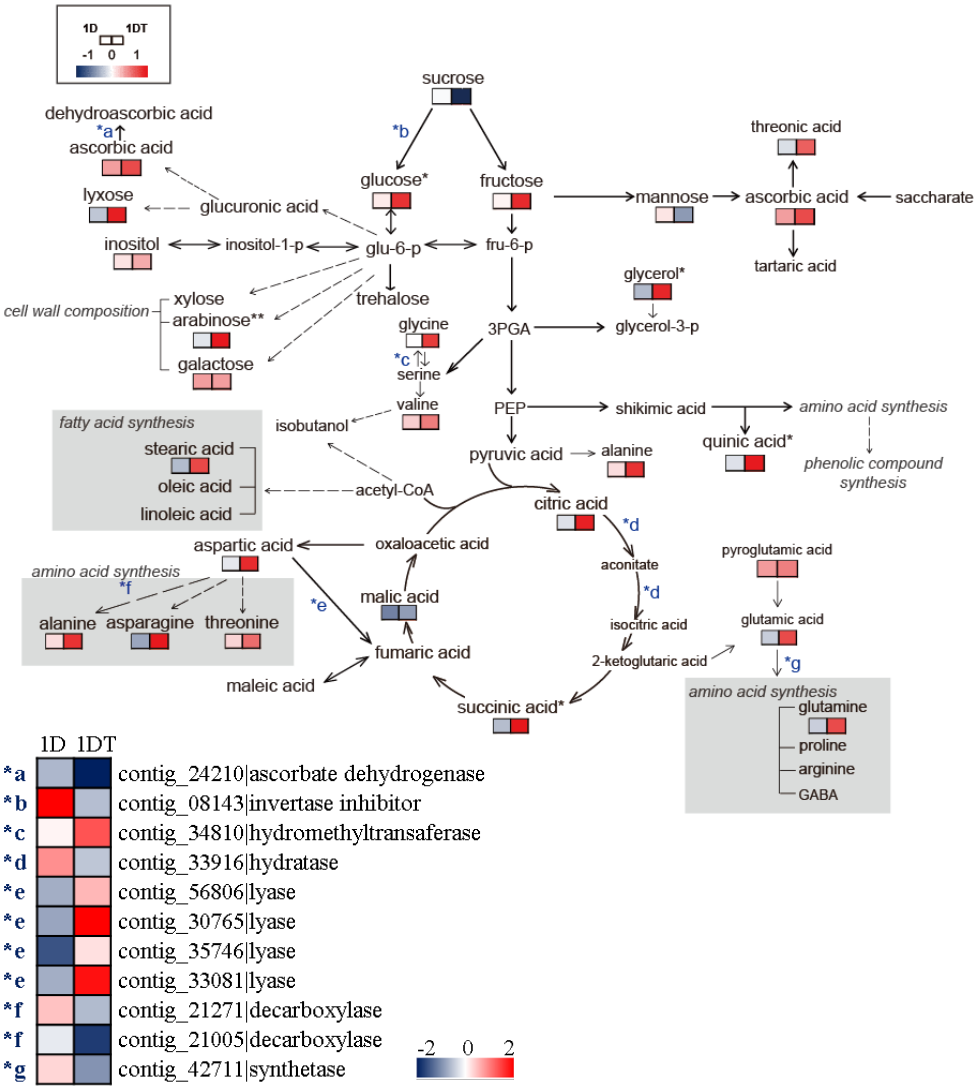


Fig. 8. Mapping GC-MS-based polar metabolite pathway. Fruits were stored at 10°C for 10 days after exposure to air (control) or 30% CO_2 for 3 h, respectively. The color scale of metabolite was calculated with normalized data used for PLS-DA. DEGs of '1D *vs* 1DT' involved in the presented metabolic pathway were presented on a heatmap. Left and right pixel means '0D *vs* 1D' and '0D *vs* 1DT' $\log_2$ fold change for each contig, respectively, according to the color scale.

Short-term 30% CO_2 maintains cell wall ultrastructure.

Fig. 9 presents the cell wall ultrastructure of strawberry fruit. The middle lamella was disintegrated and degraded in controls, and empty space between cell walls was observed after 5 days of storage (**Fig. 9B**). Meanwhile, the middle lamella of treated fruit was maintained (**Fig. 9C**), and cell walls between adjacent cells remained attached after 5 days (**Fig. 9D**).

Pectin is the main component of the cell wall and its degradation is a major cause of fruit softening (Fischer and Bennett, 1991). Because CO_2 maintained fruit firmness (**Fig. 1C**), we examined the pectin contents of cell walls. The galacturonic acid content was highly maintained in CO_2-treated fruits (**Fig. 10**). After 3 days, the levels were 18.4 and 26.9 $mg \cdot g^{-1}$ dry weight in controls and treatment fruits, respectively.

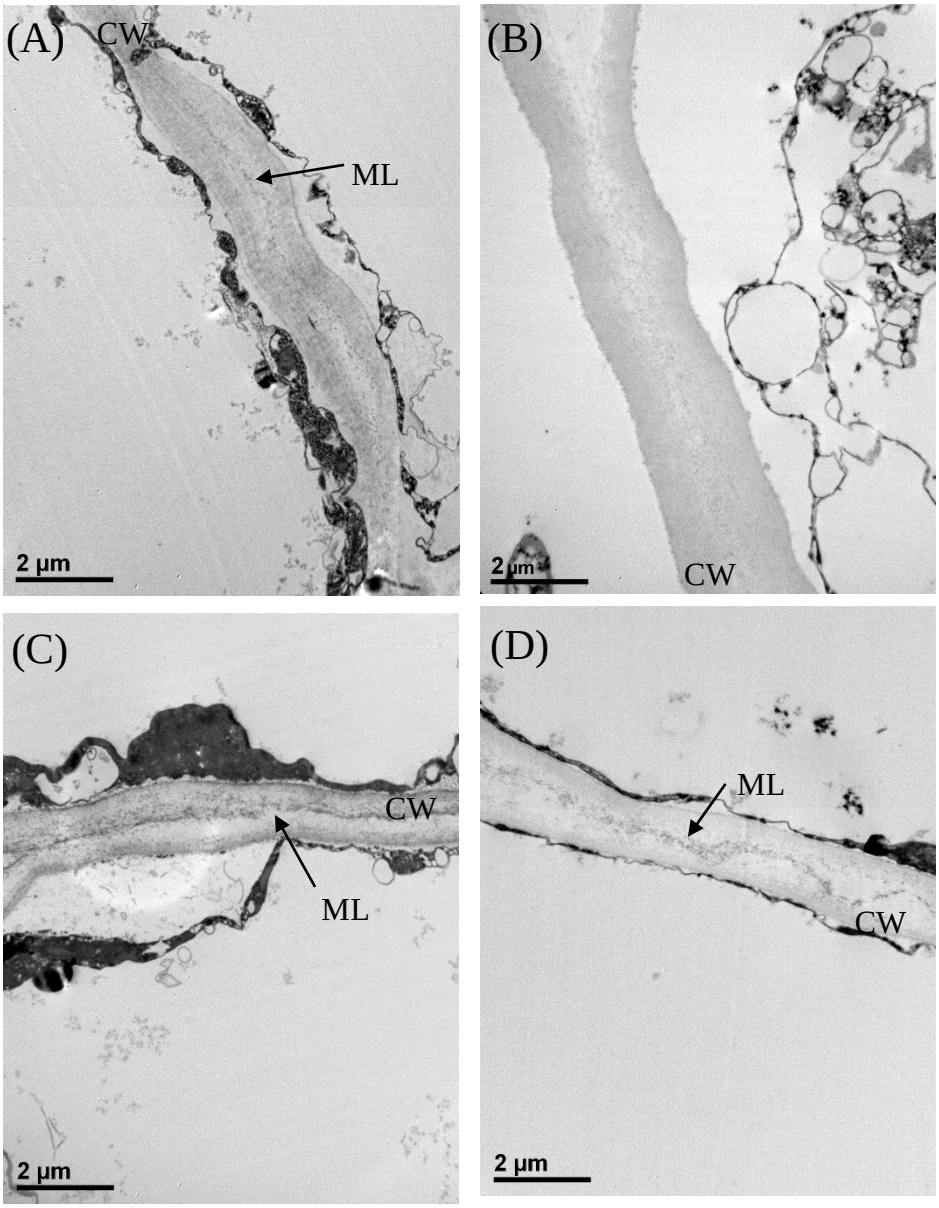


Fig. 9. TEM images of harvested strawberry fruits. Fruit were stored at 10°C for 10 days after exposure to air (control) or 30% CO_2 for 3 h, respectively. Image show control sample at 3 days (A), control sample at 5 days (B), 30% CO_2 treated sample at 3 days (C), and 30% CO_2 treated sample at 5 days (D), respectively. Abbreviations are CW (cell wall) and ML (middle lamella).

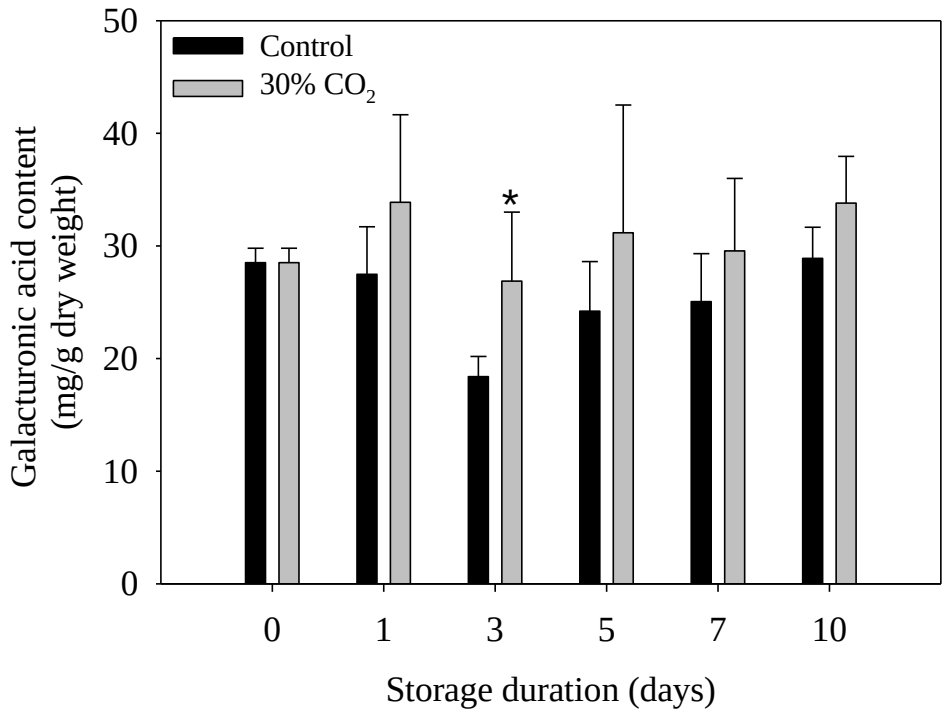


Fig. 10. Polyuronides content of harvested strawberry fruit. Fruits were stored at 10ºC for 10 days after exposure to air (control) or 30% CO_2 for 3 h, respectively. Cell wall were extracted and galacturonic acid contents were determined by colorimetric assay. Data are means of 3 biological replicates ± standard deviations (n=3 for each replicate). * represents a significant difference at $p < 0.05$.

DISCUSSION

It was obvious that the firmness of the strawberry fruit was maintained and the decay of fruit was suppressed by 30% CO_2 (**Fig. 1**). This study provides a first investigation of the cellular responses of strawberry fruit exposed to short-term 30% CO_2 after harvest based on transcriptomic and metabolomic analysis.

Fruits may have recognised CO_2 exposure as an abiotic stress, because abiotic responses induced by CO_2 exposure were observed in the DEG map in MapMan (**Fig. 6**). Along with DEGs related to pathogen/pest recognition and signal transduction, many DEGs were related to abiotic stress, and putative involvement of biotic stress was observed.

The expression of HSP genes, known as abiotic stress (e.g., heat, cold, drought, and light) responsive genes, was significantly upregulated by CO_2 treatment (**Fig. 5D-G**), but pathogen defence related gene expression was not affected (**Fig. 5H-M**). Various HSPs participate in protein–protein interactions and posttranslational modification, enhancing abiotic stress tolerance in plants (Timperio *et al.*, 2008). Pathogenesis-related proteins are related to biotic stresses such as those induced by fungi, bacteria, viruses, and viroids (Stintzi *et al.*, 1993). Strawberry fruit likely recognises short-term high CO_2 as an abiotic stress, rather than a biotic stress, and then responds by changing its responsiveness to CO_2.

Similarly, metabolite analyses revealed an abiotic response in CO_2-treated fruit. Plant photosynthesis is inhibited under abiotic stress (e.g., temperatures of 35°C or 25°C). Such abnormal conditions decrease the net photosynthetic rates in St. John's wort (*Hypericum perforatum*, cv. 'Topas')(Zobayed *et al.*, 2005). Under stress, plants rapidly invert polysaccharides into monosaccharides via invertase to produce metabolites required for defence (Liu *et al.*, 2016). The sugar metabolism of CO_2-treated fruit exhibited a similar response, with decreased sucrose levels and increased glucose and fructose levels, the two sub-units of sucrose (**Fig. 8**). Transcriptome analyses indicated that CO_2 exposure downregulated invertase inhibitor (XM_004292618.2) (**Fig. 8**). Alteration of sucrose metabolite and invertase expression levels may be a component of the abiotic response in strawberry fruit.

CO_2 exposure increased the metabolite contents associated with plant defence. Defence-related stimulation may have occurred in the '1DT' samples, because fruit decay was inhibited. CO_2 exposure did not stimulate defence-related gene expression (**Fig. 5H–M**); however, metabolites of amino acids related to the defence response increased in '1DT' (**Fig. 8**). Amino acids are the major form of plant nitrogen and are involved in plant defence. Hypersensitive and pathogen-resistance responses involve amino acid metabolism, exposure to reactive oxygen species, and salicylic acid pathways. Amino acids such as glutamine, glutamic acid, ornithine, proline, aspartic acid, threonine, methionine, and isoleucine are related to plant defence responses (Rojas *et al.*, 2014). In metabolite analyses, accumulation of several amino acids (e.g., asparagine, aspartic acid, threonine, glutamic acid, glutamine, alanine, and glycine)

was increased in CO_2-treated fruits (**Fig. 8**) compared to controls, and could be involved in the strawberry fruit defence response to 30% CO_2.

Quinic acid was significantly increased in CO_2-treated fruits (**Fig. 8**), with the second highest VIP score (**Fig. 7C**). Strawberries usually undergo a decrease in acidity during ripening (Azodanlou *et al.*, 2004), mainly due to decrease in citric acid, malic acid, and quinic acid content (Sistrunk and Cash, 1973). Because CO_2 treatment delayed ripening, there were significantly higher amount of quinic acid in treated fruit. Quinic acid is a constituent of chlorogenic acid, which is formed via the esterification of cinnamic acid derivatives and quinic acid (Gonthier *et al.*, 2003). Mhlongo *et al.* (2014) demonstrated that microbe-associated molecular pattern molecules (e.g., flagelin-22 and chitosan) elicited chlorogenic acid as a priming reaction in cultured tobacco cells. Moreover, chlorogenic acid shows antioxidant activity in plants (Takahama and Oniki, 1997). Here, high quinic acid concentrations may have supported the reduced decay of strawberry fruit triggered by 30% CO_2 (**Figs. 1A and B**).

CO_2 exposure delayed cell wall degradation by maintaining the integrity of the middle lamella (**Fig. 9**), which was less dense in control fruits after 5 days (**Fig. 9B**). Middle lamella, which adhere the cell walls of adjoining plant cells, are usually degraded during ripening in apple (*Malus sylvetris* Mill), pear (*Pyrus communis* L.), and tomato (*Solanum lycopersicum*)(Ben-Arie *et al.*, 1979), becoming empty or separated after degradation (Brummell and Harpster, 2001). Middle lamella degradation is affected by polygalacturonase and pectinesterase (Brummell and

Harpster, 2001). Pectin constitutes about 60% of the cell wall in fruit, and is the main component of the middle lamella. Pectins have a galacturonic acid chain backbone and are classified (e.g., homogalacturonan, rhamnogalacturonan, and xylogalacturonan) according to their branched side chains (Vincken *et al.*, 2003). Molina-Hidalgo *et al.* (2013) transiently silenced putative rhamnogalacturonate lyase, a pectin-degrading enzyme. They found that silenced strawberries showed a clear and dense middle lamella structure, whereas middle lamella degradation was observed in non-silenced fruit. Our result are coherent with this observation, as we measured down-regulation of the cell wall degradation enzyme pectinesterase by 30% CO_2 and the inhibition of cell wall degradation in the middle lamella of strawberry fruit (**Fig. 5B**).

Pectin concentrations in the crude cell wall between the control and treatment groups correlated with fruit firmness (**Fig. 1C**). Differential expression of genes involved in cell wall degradation underlined the close relationship between metabolomic and transcriptomic changes (**Fig. 5A–C**). The pectin homogalacturonan is released in a highly methyl-esterified form and undergoes dimethyl-esterification via pectinesterase in tomato fruit (Koch and Nevins, 1989; Hall *et al.*, 1993). The activity of polygalacturonase, which catalyses the hydrolytic cleavage of pectin, is affected by pectinesterase activity in tomato cell walls (Pressey and Avants, 1982). Moreover, minimally (33%) methyl-esterified pectin is partially depolymerised but highly (74%) methyl-esterified pectin is less degraded by polygalacturonase (Wakabayashi *et al.*, 2003). Pectinesterase helps degrade highly methyl-esterified

pectin by reducing methyl-esterification. Overall, pectinesterase activation causes pectin demethylation, and polygalacturonase can degrade pectin into small compounds (Brummell and Harpster, 2001). In treated strawberry fruit, the downregulated expression of pectinesterase under CO_2 treatment likely allowed methyl-esterified pectin to remain, preventing polygalacturonase from cleaving the pectin chains in cell walls. Hence, pectin levels remained higher in treated fruits than in controls (**Fig. 10**).

CO_2 treatment enhanced fruit storability via the activation of abiotic stress-related genes (e.g., HSPs) and deactivation of genes related to cell wall degradation enzymes (e.g., expansin, pectinesterase, and β-xylosidase). We concluded that strawberries recognise 30% CO_2 as an abiotic stress, stimulating tolerance mechanisms. Similar approaches showing enhanced strawberry fruit storability using stress stimuli have been reported. For example, heat (45°C for 3 h in air) delays postharvest softening of strawberry fruit via the downregulation of the expression of genes associated with cell wall metabolism (Martínez and Civello, 2008). Meanwhile, heat (45°C for 3 h in air) enhances antioxidant capacity by increasing enzyme activity, which is related to oxidative metabolism (e.g., pyrogallol peroxidase, superoxide dismutase, and ascorbate peroxidase)(Vicente *et al.*, 2006). In apples, short-term environmental stress (low O_2 levels and high CO_2 levels) improves postharvest quality during storage (Johnston *et al.*, 2002). As a postharvest technology, a mild external stimulus could help prolong the storability of strawberry fruit, and treatment with short-term 30% CO_2 represents an economic and efficient method.

This study documents the transcriptomic and metabolomic changes that occur in strawberry after exposing the fruit to 30% CO_2. Further investigation about the cellular responses and molecular mechanisms induced by 30% CO_2 are helpful for improving or developing eco-friendly postharvest technology and providing basic knowledge to identify genes that are highly responsive to CO_2 to breed strawberry cultivars with better storability.

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초 록

딸기 과실의 저장성을 증진시키기 위해 수확 한 과실에 30% 이산화탄소를 3시간 처리하는 수확 후 관리기술이 적용되고 있다. 본 연구에서는 단시간 30% 이산화탄소 처리에 의해 유도되는 딸기의 새로운 세포 반응을 전사체와 대사체 분석을 통해 구명하였다. 논산에서 수확 한 '설향' 품종의 딸기 과실을 대조구로 일반 대기 가스를, 처리구로 30% 이산화탄소 가스를 각각 3시간 동안 처리한 후 10°C의 저장고에 10일 간 저장하였다. 그 결과 30% 이산화탄소가 딸기 과실의 경도 유지와 부패 억제에 효과가 있음이 확인되었다. 전사체 분석을 통해 30% 이산화탄소에 의해 세포벽 분해와 관련된 expansin, pectinesterase, β-xylosidase의 발현이 억제되고, heat shock protein의 발현이 증가하는 것이 확인되었다. 대사체 분석의 경우, 30% 이산화탄소에 의해 glucose, quinic acid, succinic acid, arabionose가 유의적으로 증가하는 것이 확인되었다. 이는 30% 이산화탄소에 의해 딸기의 숙성이 지연되었기 때문일 것이라 추측된다. 전자 투과 현미경을 통해 30% 이산화탄소가 딸기의 중간 박막층의 분해가 지연됨을 확인하였다. 중간 박막층은 식물 조직에서 마주하는 세포를 서로 접착하는 부위인데, 30% 이산화탄소에 노출 된 딸기의 경우 중간

박막층이 와해되지 않고 선명하게 유지됨이 확인되었다. 저장 3일 차에 30% 이산화탄소에 노출된 딸기 과실의 세포벽 내의 pectin의 함량은 일반 대기 가스를 처리한 딸기 과실의 펙틴 함량보다 46% 높게 존재하는 것이 확인되었다. 본 연구를 통해 30% 이산화탄소가 딸기 과실의 세포벽 분해 효소의 발현을 억제하여 세포벽의 와해를 지연시키고 비생물학적 스트레스에 대항하는 유전자의 발현을 유도하여 딸기 과실의 저장성을 높이 것을 확인하였다.

주요어: 이산화탄소, 설향, 전사체, 대사체, 식물세포벽, 수확후관리
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