Research Article

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Physicochemical properties and transcriptional changes underlying the quality of 'Gala' apples (*Malus* × *domestica* Borkh.) under atmosphere manipulation in long-term storage

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Abstract

BACKGROUND: The year-round availability of apples (*Malus* × *domestica* Borkh.) depends on post-harvest technologies, which are essential for the retention of fruit sensory and chemical properties by delaying senescence. The effectiveness of strategies for preserving the quality of apples depends on complex interactions between the storage environment and endogenous biological factors. In the current work, we integrated instrumental, sensory, and transcriptional data to determine the role of conservation technologies cold storage, controlled atmosphere, and 1-methylcyclopropene-mediated ethylene blockage on the long-term conservation of apples.

RESULTS: The results demonstrated that inhibition of the consumer's perception of the apples' ethylene content is essential for long-term cold storage, and such quality conservation can be achieved by reducing oxygen pressure. Overall appreciation of apples after storage was determined mainly by their texture, with crispness and juiciness contributing favorably, and mealiness contributing negatively. Reduced oxygen pressure and inhibition of ethylene perception exerted distinct effects on the transcription of candidate genes associated with ripening in apple. Hexose and cell-wall carbohydrate metabolism genes exhibit distinct expression patterns under storage.

CONCLUSION: Inhibition of ethylene perception and reduction of relative oxygen pressure under cold storage both promote similar conservation of apple sensory traits under long-term cold storage. Texture was the main contributor to global appreciation of apples subjected to long-term storage. The conditions that were investigated were able to delay, but not fully prevent, senescence, as evidenced by physicochemical and gene expression analyses. The expression of gene-encoding enzymes involved in hexose metabolism was mainly developmentally regulated, whereas storage conditions exerted a stronger effect on the expression of genes associated with cell-wall metabolism.

Supporting information may be found in the online version of this article.

Keywords: 1-Methylcyclopropene; controlled atmosphere; fruit quality; gene expression; multivariate analysis

INTRODUCTION

The apple (*Malus x domestica* Borkh.) is an economically important fruit for *in natura* consumption, mainly due to its attractive sensory and functional properties, including taste, flavor, texture, and phytochemicals.[1,2] Apples are the third most commonly produced fruit worldwide, with a global production of 86 million tons in an area of 46 000 ha.[3] Since 2011, the yields in South America have increased steadily, despite the decrease in planted area, mainly due to technological advances.[3] The availability of high-quality apples to the market throughout the year is a critical factor for the economic viability of the productive chain. Post-harvest

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conservation techniques are therefore often under close investigation and are often adjusted to maximize their effectiveness.

Physiologically, apples are classified as climacteric fruits that show a steep and concomitant increase in respiration and ethylene biosynthesis during the final developmental stages of ripening.[4,5] To delay senescence, apples are conserved under specific controlled conditions, mainly by the manipulation of the storage temperature and atmosphere.[6,7] Conservation technologies employ cold storage (CS) under a regular or controlled atmosphere (CA) or a dynamically controlled atmosphere (DCA), often combined with the application of gaseous substances to specifically block the action of ethylene, the main hormone controlling ripening.[7–9]

Developmental and metabolic changes triggered by ethylene are the most important biological changes for climacteric fruits.[10,11] Thus, post-harvest technologies that aim to reduce hormone perception, signal transduction, and production are frequently employed.[7–9,12] Ethylene competitive inhibitor 1-methylcyclopropene (1-MCP) prevents the hormone from binding to its cellular receptors,[8,12] and is frequently used to conserve quality of horticultural products after harvest.[12]

Reduced levels of cellular oxygen limit mitochondrial respiration in a condition called hypoxia.[13] The responses to hypoxia in fruit tissues include a reduction in cellular respiration and ethanol biosynthesis, followed by the induction of anaerobic respiration, affecting the transport of metabolites and several energy-associated pathways, including glycolysis, the cycle of tricarboxylic acids (TCA) and the metabolism of amino acids.[13] The reduction in cellular levels of O_2 also affect ethylene production and tissue sensitivity negatively.[13,14] As a consequence of the modifications in fruit physiological and metabolic processes, hypoxia delays ripening and senescence of fruit.[14,15] However, the levels of O_2 restriction have to be carefully balanced to prevent senescence without causing physiological disorders leading to undesirable marketing characteristics.[13–15]

Apple sensory and physicochemical traits can be preserved for up to 12 months after harvest, depending on the genotype, cultural practices, harvest point, and storage conditions.[15,16] The effectiveness of post-harvest technologies in preserving apple shelf life depends on complex interactions between environmental and biological factors, and the molecular changes underlying the later ripening stages under conservation conditions are not fully understood.

The pleiotropic activity of ethylene in plant development[17] and the distinct interaction networks present in different genomic contexts influence the effectiveness of atmosphere manipulation in apple conservation.[7–9,15,16] The inhibition of ethylene responses after harvest has been associated to transcriptional and metabolic reprograming leading to undesirable traits and physiological disorders.[8,12,16,18]

The changes induced by senescence after harvest and/or triggered by conservation technologies are controlled by several genome regions. They are often perceived negatively by consumers.[19,20]

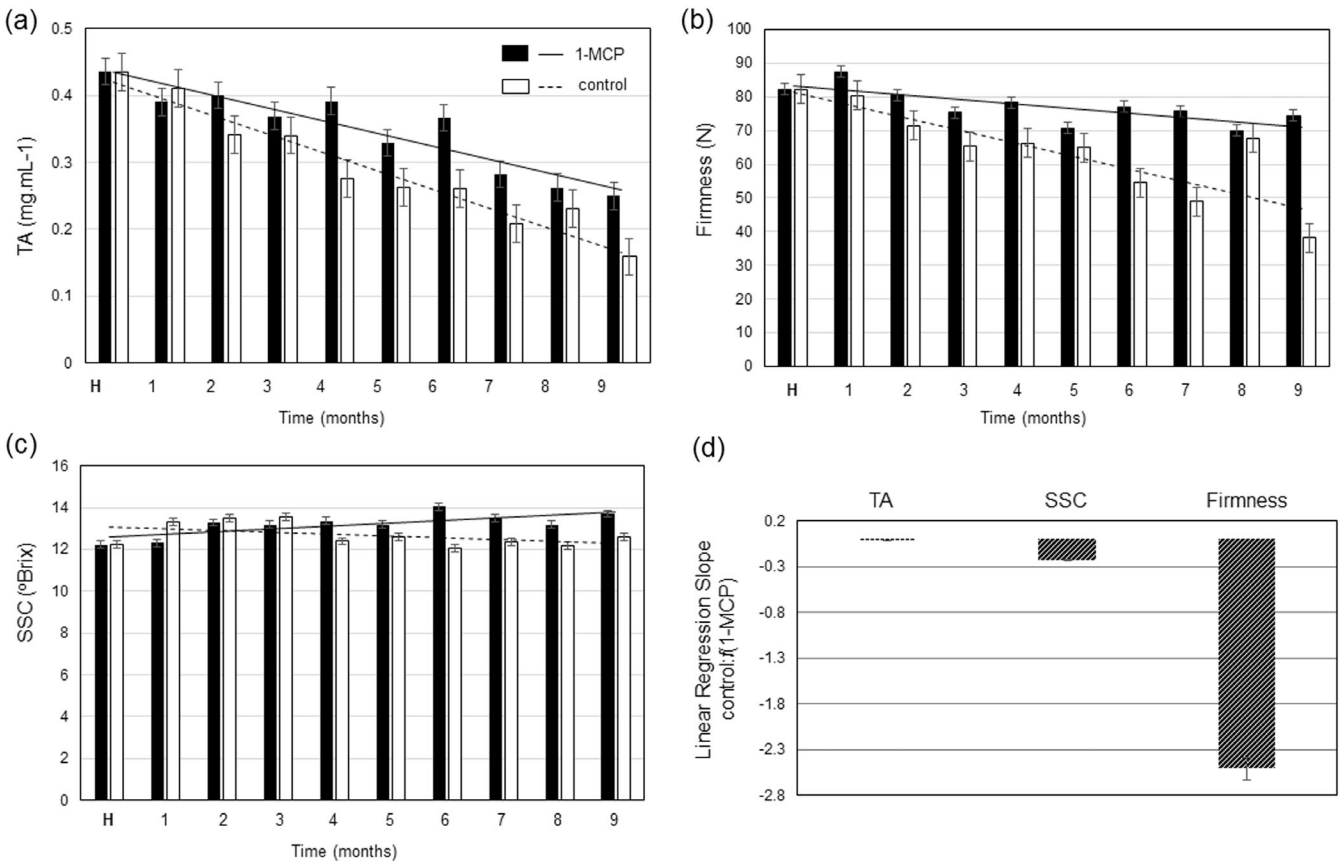

Figure 1. Changes in physicochemical characteristics of apples under long-term cold storage (CS). The fruit quality traits – titratable acidity (TA) (**a**), solids contents (SSC) (**b**), flesh firmness (F) (**c**) – were determined at harvest (H) and during post-harvest conservation, in the absence (control) and presence of 1-MCP application (1-MCP). The significance of 1-MCP application is shown as linear regression slope comparison (**d**) for TA, SSC, and firmness. Average and standard deviation (sd) data for each data point are presented in Table S2 in the supporting information. Linear models are shown and their statistical significance is provided in Table S3 in the supporting information.

Taste and texture attributes are considered the most critical factors driven acceptance of apples by the consumer.[19,20] However, the interpretation of data from instrumental, sensory, and molecular analysis is challenging, and requires sophisticated statistical approaches.[21] The association between sensory data and their corresponding instrumental measurements is not always straightforward[22] and suffers influences from several unaccounted-for factors, such as the release of volatiles and visual properties.[19]

Previously, we demonstrated that inhibition of ethylene perception underlies major transcriptional changes in apple under CS and CA, promoting a partial reappraisal of anabolic pathways;[15,18] however, the extent of these molecular changes on the consumers' perception of stored apples remains uncharacterized. To address this question, we integrated extensive instrumental and sensory characterization of apples during long-term storage under CS and CA with expression profiling

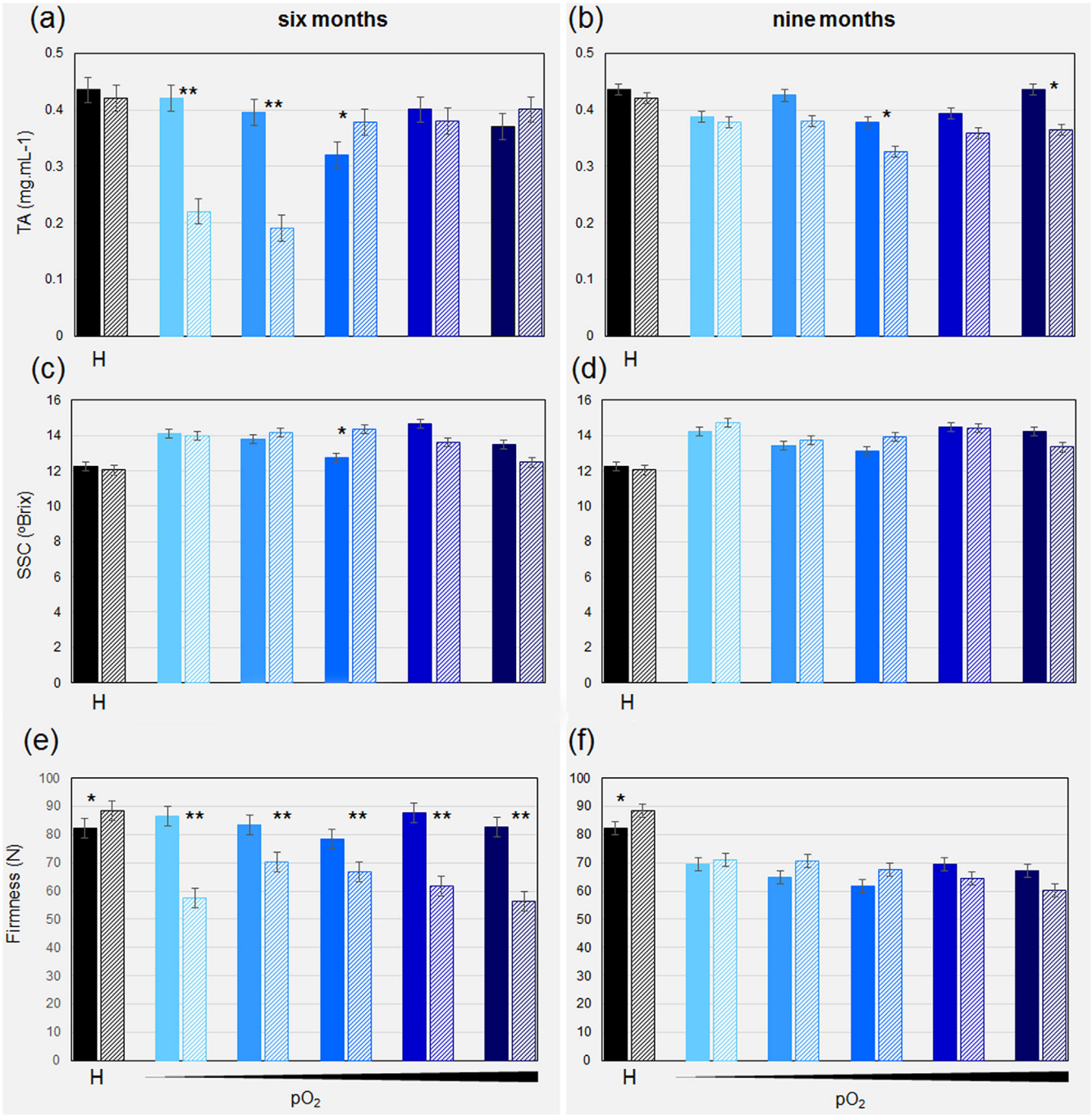

Figure 2. Changes in physicochemical characteristics of apples stored under controlled atmosphere (CA) for six and 9 months. Fruit quality traits titratable acidity (TA) (**a** and **b**), solids contents (SSC) (**c** and **d**), and flesh firmness (F) (**e** and **f**) were determined at harvest (H), at 6 and 9 months under CA, in the absence (control) and presence of 1-MCP application (1-MCP). Relative O_2 pressure (0.3, 0.4, 0.5, 1.0, and 1.5 kPa) in the storage chambers is presented as a blue gradient and, schematically on the *x*-axis. Statistical significance of differences between control and 1-MCP-treated samples at each time point is represented by * and ** for $P \leq 0.05$ and 0.01, respectively. Average and standard deviation (sd) data for each data point are presented in Table S2 in the supporting information.

of candidate genes associated with later ripening and senescence developmental processes.

MATERIAL AND METHODS

Plant material

Apples (*Malus* × *domestica* Borkh., cv. Gala, clone Baigent) were harvested from random trees in a commercial orchard in Caxias do Sul, Rio Grande do Sul, Brazil (29° 09′ 36.39″ S, 50° 54′ 27.33″ E), at physiological maturity determined by firmness, total solid soluble, titratable acidity, and iodine-starch indices.[23] The bulk amount of fruit (approximately 900 fruit, or 150 kg) was sub-divided equally into the treatments, as schematically represented in Fig. S1 in the supporting information. Initially, the apples were sub-sampled into control and 1-methylcyclopropene (1-MCP) treated, and they were further randomly distributed among the storage conditions (as detailed in Fig. S1 in the supplementary information). Each data point was obtained from three independent replicates consisting of at least 15 fruit (approximately 3 kg of apple) per sampling point.

Ethylene inhibitor treatment and storage conditions

Fruits were treated with 1 μg L^{-1} SmartFresh® (Agro Fresh, Rohm and Haas, PA, USA), consisting of 0.14% 1-MCP,[24] for 24 h in a hermetically sealed recipient (capacity 293 L, Marfinite®, Model TP1034) at 20 °C. After treatment, control and treated apples were transferred immediately to the storage conditions.

The apples were stored at a room temperature (RT) of 20 ± 5 °C for 7 days or under cold storage (CS) (0–0.5 °C, 90–95% relative humidity) for 9 months, or under a controlled atmosphere (CA) (0–0.5 °C, 90–95% relative humidity) with distinct partial O_2 pressures (pO_2) of 1.5, 1.0, 0.5, 0.4 and 0.3 kPa combined with 2.0 kPa CO_2 for 9 months (Fig. S1 in the supporting information). Temperature and humidity were monitored daily using digital thermometers and humidity sensors inside the chambers. Experimental mini-chambers (capacity 106 L), located inside a 0 °C cold chamber, were set to the investigated O_2 pressure in combination with 2.0 kPa pCO_2. Atmosphere manipulation was carried out by flushing the mini-chambers with gaseous nitrogen until the desired oxygen pressure was reached. Oxygen and carbon dioxide concentrations were monitored and corrected, as necessary, by the CA control system (Besseling CA Systems, Holland, Heerhugowaard). The equipment is coupled to a gas analyzer (System BatNetWin 16.20, Besseling CA Systems) that controls O_2 injection and CO_2 removal with a scrubber.

The fruit submitted to CS and CA were analyzed immediately after removal from storage and after a 7 day recovery period at RT, as used commercially. Storage conditions and fruit sampling points are summarized in Fig. S1 in the supporting information.

Instrumental analyses

Physicochemical characterization was performed by determination of the soluble solids content (SSC), titratable acidity (TA) and flesh firmness (F), as described.[16] Fruit firmness (N) was measured on opposite sides of the apples after peel removal, using an electronic texture analyzer (Fruit Texture Analyzer, FTA, Güss, Strand, South Africa), equipped with an 11 mm tip. The SSC was analyzed employing a refractometer (PR 101, Atago, São Paulo, Brazil) (0–45%) with temperature correction. Acidity was determined by titrating 10 mL of juice diluted in 90 mL of distillated water with 0.1 N NaOH to an end point of pH 8.1. Total acidity is expressed as mg equivalents of malic acid per 100 mL of juice.

Table 1. Individual and interaction effects of ethylene blockage (1-MCP), resting period at room temperature (7 days RT), storage time (mo^{-1}), and partial O_2 pressure (pO_2) at harvest (H), during cold storage (CS) or controlled atmosphere (CA) on the physicochemical and sensory properties of apples stored for 9 months. The effect of time under storage is considered for CS and CA, and is expressed as monthly changes in comparison to the values at H. Mean values at H are followed by residual standard errors (RMSE of the model). The remaining values are expressed as deviations (Δ) from H value, followed by statistical significance (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS = not significant)

	Physicochemical			Sensory						
Source of variation	TA (% malic acid)	SSC (°Brix)	Firmness (N)	Aroma	Sweetness	Acidity	Crispness	Juiciness	Mealiness	Global appreciation
Harvest	0.385 ± 0.051	13.01 ± 0.76	84.9 ± 10.9	2.09 ± 1.82	5.62 ± 1.65	4.49 ± 1.70	5.91 ± 1.63	6.16 ± 1.54	3.54 ± 1.81	6.58 ± 1.70
1-MCP (Δ)	NS	NS	NS	NS	NS	NS	+0.99***	NS	−0.75*	NS
7 days RT (Δ)	−0.056*	NS	−4.54*	+2.69***	NS	NS	NS	NS	+1.10***	NS
1-MCP + 7days (Δ)	NS	NS	NS	−1.90***	NS	+1.01*	+0.87*	NS	NS	NS
CS (Δ.mo^{-1})	−0.026***	NS	−4.51***	+0.25***	NS	−0.15***	+0.23***	−0.15***	+0.21***	−0.20***
1-MCP (Δ.mo^{-1})	+0.013**	+0.19**	+2.58***	NS	NS	+0.12*	+0.15**	NS	NS	+0.16**
7 days RT (Δ.mo^{-1})	NS	NS	NS	−0.38***	NS	NS	NS	−0.13*	NS	NS
1-MCP + 7days (Δ.mo^{-1})	NS	NS	NS	+0.32***	NS	NS	NS	NS	NS	NS
CA (Δ.mo^{-1})	NS	+0.11***	−0.31*	NS	NS	+0.18***	+0.15***	NS	−0.12***	NS
1-MCP (Δ.mo^{-1})	NS	NS	NS	NS	NS	−0.11*	−0.21***	NS	+0.16***	NS
7 d RT (Δ.mo^{-1})	NS	NS	NS	−0.40***	−0.13**	NS	NS	NS	−0.15**	NS
1-MCP + 7days (Δ.mo^{-1})	NS	NS	NS	+0.21**	NS	NS	NS	NS	NS	NS
pO_2 (Δ.mo^{-1}.kPa^{-1})	NS	NS	−0.70***	NS	+0.08***	−0.07**	−0.05*	+0.05*	NS	NS

Sensory evaluations

A trained panel of 15 members evaluated the fruits for aroma, texture (crispness, juiciness, and mealiness) and taste-related (sweetness and acidity) properties, along with a global appreciation grade, according to an hedonic scale from the lowest (1) to the highest perceivable intensity (9). Sensory evaluations were conducted in a dedicated laboratory, kept at 20 °C, equipped with isolation booths, and white lights. Fruit preparation was conducted in an area physically separated from the tasting booths. The purpose of the study and the details of the treatments were not disclosed to the panel.

Apples were sliced, placed under a covered plastic cup, and labeled according to a random three-digit code, up to 15 min before tasting. Slices were presented to the panel with a cup of water and unsalted crackers as palate cleansers. Panelists were instructed to swallow the samples after tasting. Sensory evaluations were repeated twice for consistency and performed according to NBR ISO 11132:2016.[24] The local ethics committee approved the experiments (CAAE number 36593814.3.0000.5305).

Gene expression profiling

Total RNA was extracted from 6 g of pulverized fruit tissue, as described.[16] The quantity and integrity of the RNA were determined by spectrophotometric readings (Epoch Micro-volume Biotek, BioTek Instruments, Inc., Winooski, VT, USA) and 1% (w/v) agarose gel electrophoresis, respectively. Two µg of total RNA were treated with DNase I (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and submitted to reverse transcription using oligo d(T) primers (Invitrogen) and Super ScriptIII/RNAse Out Mix (Invitrogen), following the manufacturer's recommendations. The absence of DNA contamination was confirmed by polymerase chain reaction (PCR) amplification, using the primer *MdH1* (*HISTONE1*) (Table S1 in the supporting information).

Primers for expression analyses were designed using Primer3Plus,[25] based on *M.* × *domestica* coding sequences (CDs) available at Genome Database for Rosaceae (GDR) (https://www.rosaceae.org/). The genes studied were *MdACO1* (*1 AMINOCYCLOPROPANE 1 CARBOXYLATE OXIDASE1*), *MdAF3* (*α L ARABINOFURANOSIDASE 3), MdEXGT (ENDO XYLOGLUCANTRANSFERASE), MdCOBRA, MdNI (NEUTRAL INVERTASE), MdSuSy (SUCROSE SYNTHASE), MdFK (FRUCTOKINASE)* and *MdHK (HEXOKINASE)*. Primer sequence and amplification information is shown in Table S1 in the supporting information.

Real-time quantitative PCR was carried out in a StepOne™ Real Time PCR system (Model LS4376357, Applied Biosystems™, Life Technologies Corporation) using SYBR™ Green PCR Master Mix (Applied Biosystems™, Life Technologies Corporation, Warrington, UK), as described.[16]

Normalizer genes were chosen from analyzing the expression profile of *MdACT* (*β-ACTIN*), *MdUBC* (*UBIQUITIN CONJUGATING ENZYME E2*), *MdPDI* (*DISULFIDE ISOMERASE*), *MdNAP1* (*NUCLEOSOME1 BINDING PROTEIN*) and *MdH1* Supporting Information (Table S1)[16] in all cDNA samples, using the DataAssist software, v 3.01 (Applied Biosystems™, Life Technologies Corporation). The most stable expression profiles were obtained for *MdACT*, *MdUBC* and *MdH1*, used as normalizers.

Statistical analyses

Statistical analyses were performed in R.[26] Instrumental and sensory data were averaged for each sampling point and used to build two auto-scaled matrices containing samples (76 and 64) and variables (physicochemical and sensory parameters). A two-way multivariate analysis of variance (two-way MANOVA) was used to investigate any possible effect of the analysis time and/or type of sensory defect perceived in the fruits, considering simultaneously all the dependent variables, according to the Pillai's trace test.

Physicochemical and sensory data were submitted to multivariate exploratory analyses using hierarchical principal component analysis (PCA) and multiple factor analysis (MFA), with atmospheric conditions during storage as continuous variables: 1-MCP treatment (0 or 1 µg L^{-1}), storage time (days), and O_2 pressure (uncontrolled O_2 pressure was considered 20.95%). Categorical variables consisted of harvest (H), room temperature (RT), cold storage (CS) and controlled atmosphere (CA). For the instrumental analyses, variables were grouped in taste- (TA and SSC) and texture-associated (F). Sensory variables were collectively classified as related to aroma (aroma), texture (crispness, juiciness, mealiness), and taste (sweetness, acidity). Global appreciation was used as a supplementary continuous variable.

Transcription profile data were integrated with storage conditions using partial least squares-discriminant analysis (PLS-DA). The performance of the PLS-DA model in classifying the samples to storage categories was assessed with fivefold cross-validation, repeated 10 times, using the packages FactoMineR,[27] factoextra,[28] and mixOmics.[29]

RESULTS

Apple physicochemical changes during long-term storage

Post-harvest technologies were not able to fully prevent ripening processes under long-term storage (Figs 1 and 2, Table 1, and Table S2 in the supporting information). Application of 1-MCP reduced acidity loss by approximately 42% in 9 months, whereas CS alone promoted even greater decrease in acid content (63.5% reduction) (Fig. 1(a)). Similarly, manipulation of O_2 pressure did not prevent acidity loss under CA (Fig. 2(a)). In comparison to 1-MCP application, CA alone reduced acidity loss under low relative pO_2 (0.3, 0.4, and 0.5 kPa), but not after 6 months under storage (Fig. 1(a), Fig. 2(a), Table 1, and Table S2 in the supporting information).

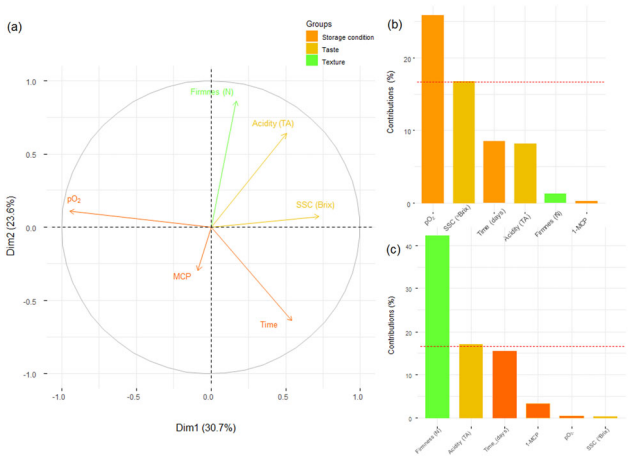

Figure 3. Multiple factor analysis (MFA) of physicochemical attributes and storage conditions to the principal dimensions (**a**) and contribution of the variables to the first (**b**) and second (**c**) dimensions. The MFA groups correspond to storage conditions (time, O_2 pressure and 1-MCP), taste (TA and SSC) and texture (Firmness) associated variables and are identified by distinct colors in the correlation and contribution plots. Dashed red lines in (**b**) and (**c**) represent the expected average value.

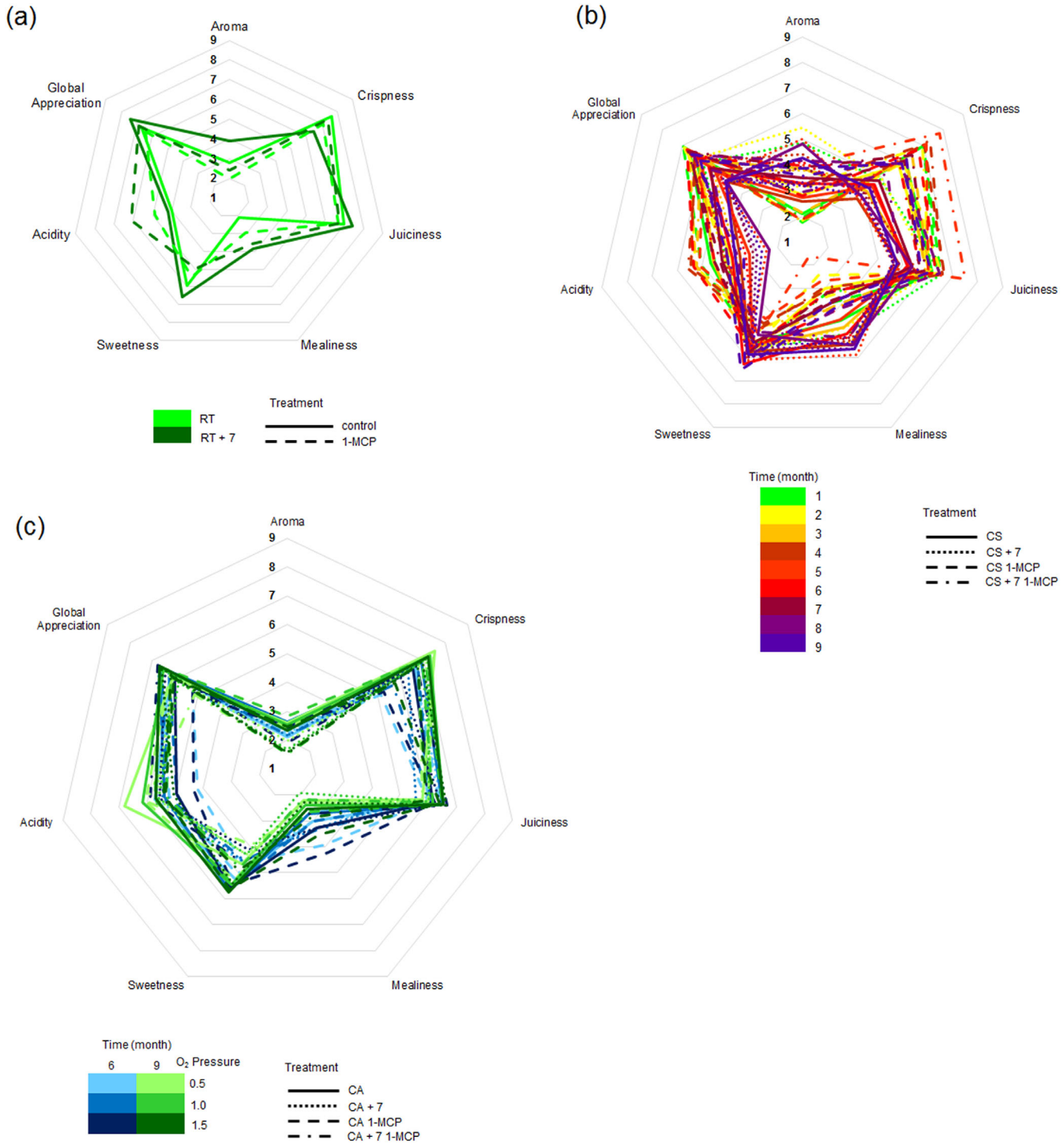

Figure 4. Sensory analysis of apples stored at RT (**a**) after harvest, CS (**b**) and CA (**c**) in the absence or presence of 1-MCP treatment. Grading system ranged from 1 to 9 and is represented as radar plots. The attributes are shown as axes. Time under storage and the rest period (7 days) are represented by color and line patterns. Partial O_2 pressure (pO_2, kPa) in CA (**c**) is shown as green gradient.

The application of 1-MCP significantly reduced firmness loss under CS, from a 53.61% to a 9.55% decrease in the absence of ethylene perception, after 9 months in storage (Fig. 1(b), Table 1, and Table S2 in the supporting information). The reduction in O_2 relative pressure alone promoted similar levels of firmness retention (15.06 to 26.48% reduction, for pO_2 of 1.0 and 1.5 kPa, respectively) after 9 months (Fig. 2(b)). The inhibition of ethylene perception did not further increase firmness retention after 9 months under CA (Fig. 2(b), Table 1, Table S2 in the supporting information).

Under CS coupled with ethylene perception blockage, the soluble solid content increased by approximately 12.0% after 9 months and CS alone reduced the accumulation of sugars to 2.8% (Fig. 1(c)). The increase in soluble solids was similar under CA (average 12.0 ± 0.40%, after 9 months), with a slight increase

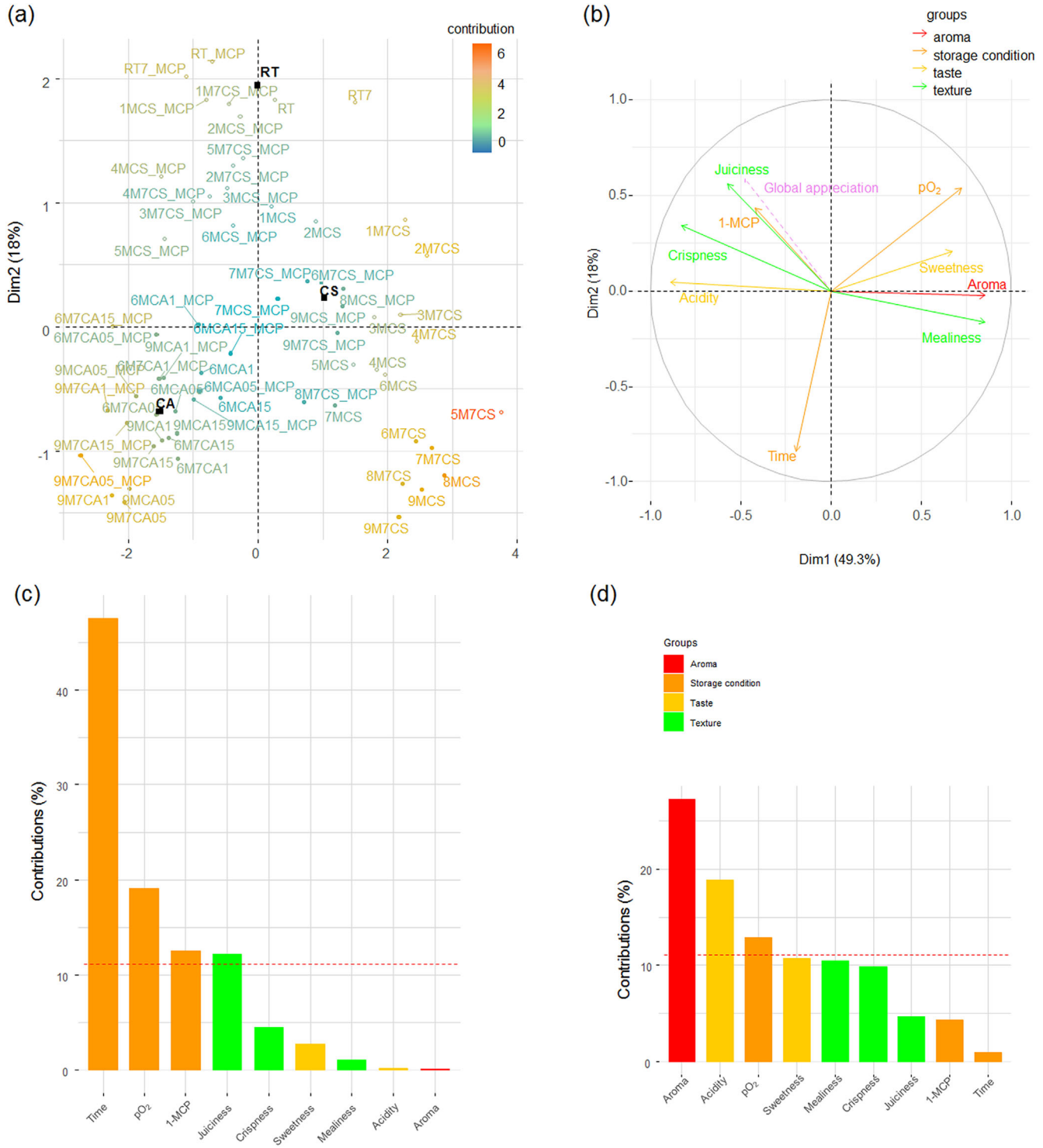

Figure 5. Individual grade contribution to apples sensory characteristics (**a**). Heatmap represents individual contribution for the treatments at each time point. Centroids of the supplemental qualitative variables representing the storage conditions RT, CS, and CA are shown in black. Correlation (**b**) and contribution to the first (**c**) and second (**d**) principal components of quantitative sensory and qualitative supplemental variables. The MFA groups consist of storage conditions (time, O_2 pressure, and 1-MCP), aroma, taste (sweetness and acidity) and texture (crispness, juiciness, and firmness) variables identified by distinct colors in the correlation plot. Contribution thresholds are represented as dashed lines.

in the presence of the ethylene inhibitor (average 16.3 ± 0.56%, after 9 months) (Fig. 2(c)). The highest tested pO_2 (1.5 kPa) had the smallest effect on the accumulation of solids (Fig. 2(c), Table 1, and Table S2 in the supporting information).

In the absence of O_2 pressure control, time under storage and ethylene perception inhibition had a significant effect, whereas under controlled atmosphere the effect of 1-MCP was negligible (Table 1, Table S2 in the supporting information).

To explore the interplay between post-harvest conservation techniques and the physicochemical changes in apple further we used multi-factor analysis (MFA), grouping the variables as: storage condition (group condition, time under storage, O_2 pressure, and 1-MCP application), taste (group Taste, TA, and SSC), and texture (group texture, firmness) (Fig. 3). A qualitative supplementary variable corresponding to cold storage (CS) and controlled atmosphere (CA) was added to the analyses. In MFA, a weight, corresponding to the first eigenvalue of the principal component analysis (PCA) for the group is attributed to each group of variables, so the global analysis is balanced, and the structure of each group is respected. Condition, taste, and texture groups explained more than half of the variance (54. 3%) (Fig. 3). Relative O_2 pressure was the storage variable that contributed the most in the first component (25.85%), whereas firmness was the most important contributor in the second component (42.23%) (Fig.3). Taste attributes contributed to the variation in the first and second components: sugar accumulation (SSC) to the first (16.77%) and acidity (TA) to the second and first (17.00% and 8.15%, respectively) (Fig. 3).

After 9 months under storage, neither conservation technology was able to preserve apple physicochemical properties fully (Fig 1 and 2). These observations are confirmed by the significant effect of time under storage (24% in total) (Fig. 3).

Apple sensory changes during long-term storage

The highest grades were given to apples kept under RT for 7 days in the absence of 1-MCP treatment (7.45 ± 0.52) (Fig. 4(a)) and the

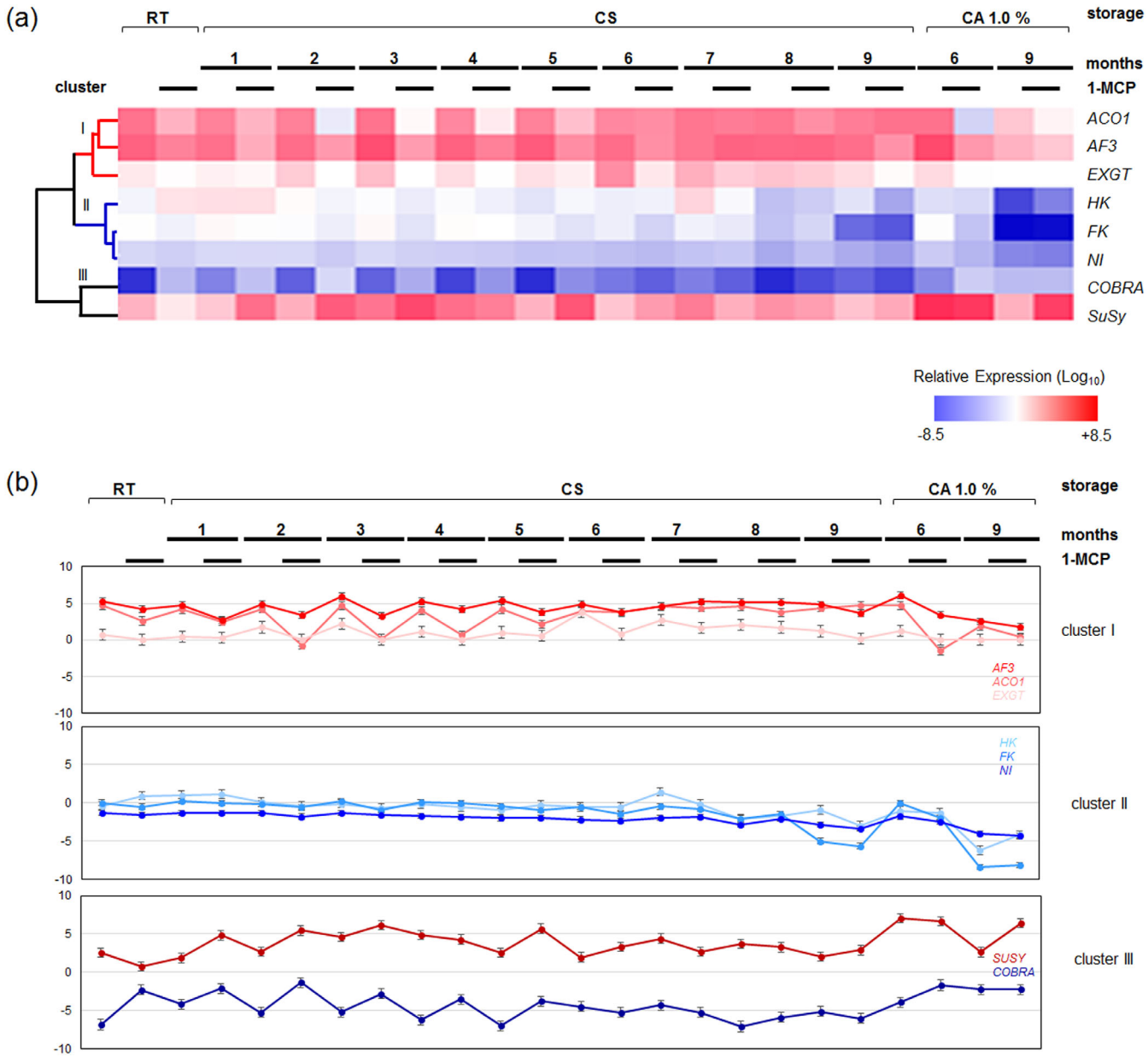

Figure 6. Expression profile of genes associated with the ripening processes in apple at RT after harvest and submitted to long-term storage under CS and CA, in the absence or presence of 1-MCP treatment (**a**). Relative expression levels are represented as a heatmap. Gene expression hierarchical clustering was performed using Euclidean distance and average linkage. Individual expression profiles for genes and standard deviation (sd) for each data point in the clusters are represented (**b**).

lowest to fruit conserved under CS for more than 7 months in the absence of 1-MCP (4.77 ± 0.08) (Fig. 4(b)). The general appreciation decreased with time under CS and CA (Fig. 4(b), (c)). Under CS, the inhibition of ethylene perception during storage increased global appreciation, improving the grades by up to 23.5% after 9 months (Fig. 4(b)). In contrast, under CA, the application of the hormone inhibitor decreased the grades of global appreciation, down to 6.67% in the lowest O_2 pressure tested (Fig. 4(c)).

Fruit aroma was the worst graded sensory feature, even for freshly harvested fruits kept under RT for 7 days (Fig. 4(a)). Treatment with 1-MCP had a negative impact on aroma grading under CS (3.55 ± 0.62, 3.73 ± 0.45) and CA (2.31 ± 0.22, 2.08 ± 0.26) (Fig. 4(b), (c)), after 6 and 9 months, respectively. However, the grades given to the apples stored under CA were approximately 38.9% lower than those given to those kept under CS, regardless of the application of 1-MCP (Fig. 4(b), (c), Table 1).

Fresh fruits, stored at RT, also received higher grades for crispness and juiciness (Fig. 4(a), Table 1). Under CS, the perception of crispness and, to a lower extent, juiciness were improved by 1-MCP treatment, especially during the first 5 months (Fig. 4(b)). After 9 months under CA, fruit kept under lower O_2 relative pressures received higher grades regardless of ethylene inhibition (7.33 ± 0.29 at pO_2 of 0.5 kPa versus 7.09 ± 0.20 at 1.5 kPa) (Fig. 4(c), Table 1). Fresh fruit and fruit conserved under CA for up to 9 months received the lower grades for mealiness (3.13 ± 0.77 and 2.49 ± 0.41, respectively) (Fig. 4(a), (c)). Mealiness perception increased approximately 15% after 9 months under cold storage. The application of 1-MCP reduced the perception of mealiness under CS, although with lower effectiveness after 5 months (Fig. 4(b), Table 1).

The blockage of ethylene signaling by 1-MCP treatment decreased sweetness and increased acidity perception in fresh apples kept under RT (Fig. 4(a)). A similar trend was observed for fruit kept under CS, especially after the fifth month (Fig. 4(b)). Interestingly, the 7 days RT rest period after removal from CA affected the perception of taste attributes, mainly after 9 months under the lowest O_2 pressures (0.5 and 1.0 kPa) (Fig. 4(c)).

In multi-factor analysis (MFA) of sensory responses, the variables were grouped as: aroma (the 'Aroma' group), texture (the 'Texture' group, consisting of crispness, juiciness and mealiness), taste (the 'Taste' group, consisting of sweetness and acidity), and storage condition (the 'Storage Condition' group, consisting of time under storage, O_2 pressure, and 1-MCP application). Global appreciation was used as a quantitative supplementary variable.

The two principal dimensions explained approximately 70% (67.3%) of the variance (Fig. 5(a)), and the distance among the variables for each storage condition clearly discriminated the storage conditions RT, CS, and CA (Fig. 5(b)). Longer storage periods under CS and CA contributed mainly to dimensions 1 and 2, although in an opposite manner (Fig. 5(b)). The variables associated with storage conditions, namely, time (41.02%), relative O_2 pressure (19.94%), and 1-MCP application (5.59%), contributed with 98.9% of the total variance of the sensory changes of the apple under conservation (Fig. 5(c)). Aroma (13.28%), taste (acidity 17.54%, and sweetness 7.98%) and texture attributes (crispness 17.93%, juiciness 19.13% and mealiness 18.33%) contributed to the variance in the two main dimensions (Fig. 5(c), (d)). The quantitative supplementary variable global appreciation correlated with variables in the texture group (0.59549123, $P = 0.0018$), including crispness and juiciness (positive correlation) and

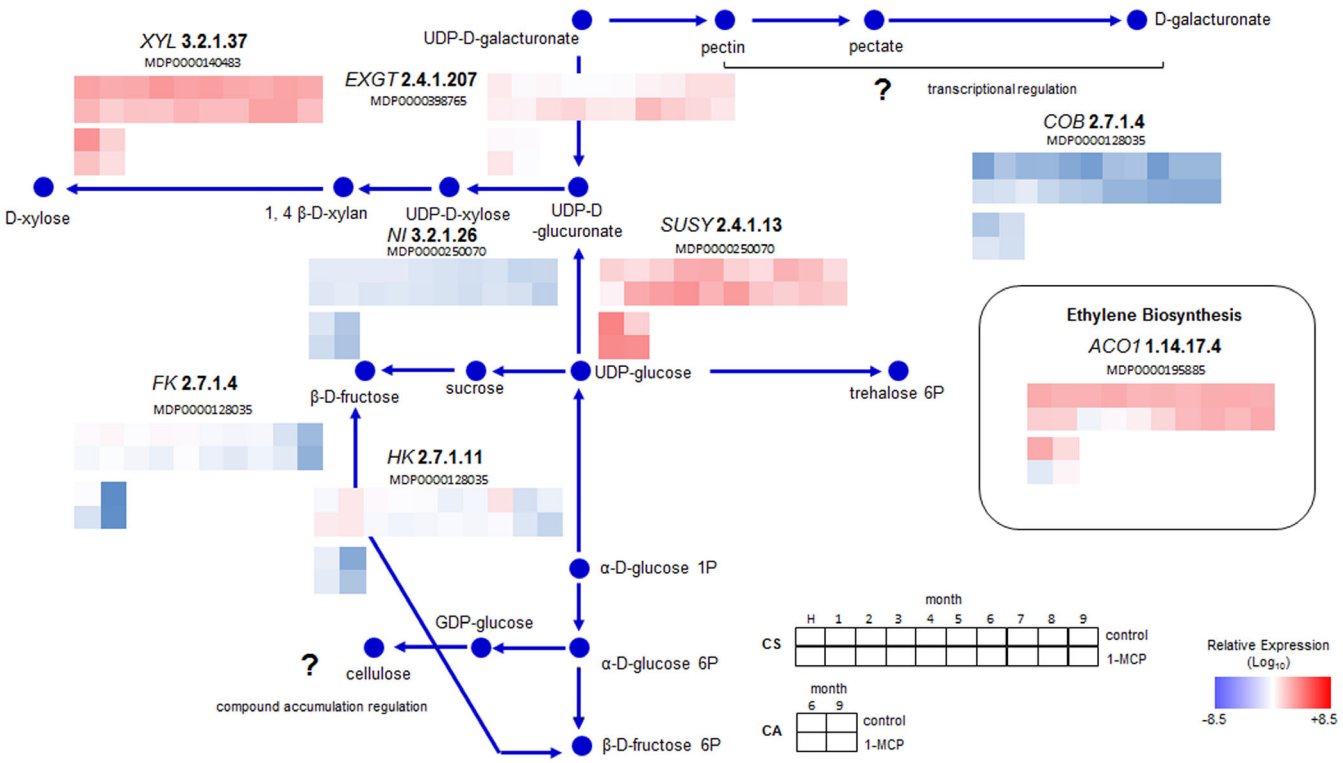

Figure 7. Relative expression levels of the genes associated with starch and sucrose metabolism and ethylene biosynthesis at harvest and during long-term storage in apple. Metabolic pathways correspond to curated maps from the Kyoto Encyclopedia of Genes and Genomes (KEGG) 00500 and 00520. Blue dots represent the metabolites and enzyme-coding sequences are accompanied by their Enzyme Commission Number (EC) and genome locus. Question marks (?) represent hypothesized functions of the gene *COBRA*.

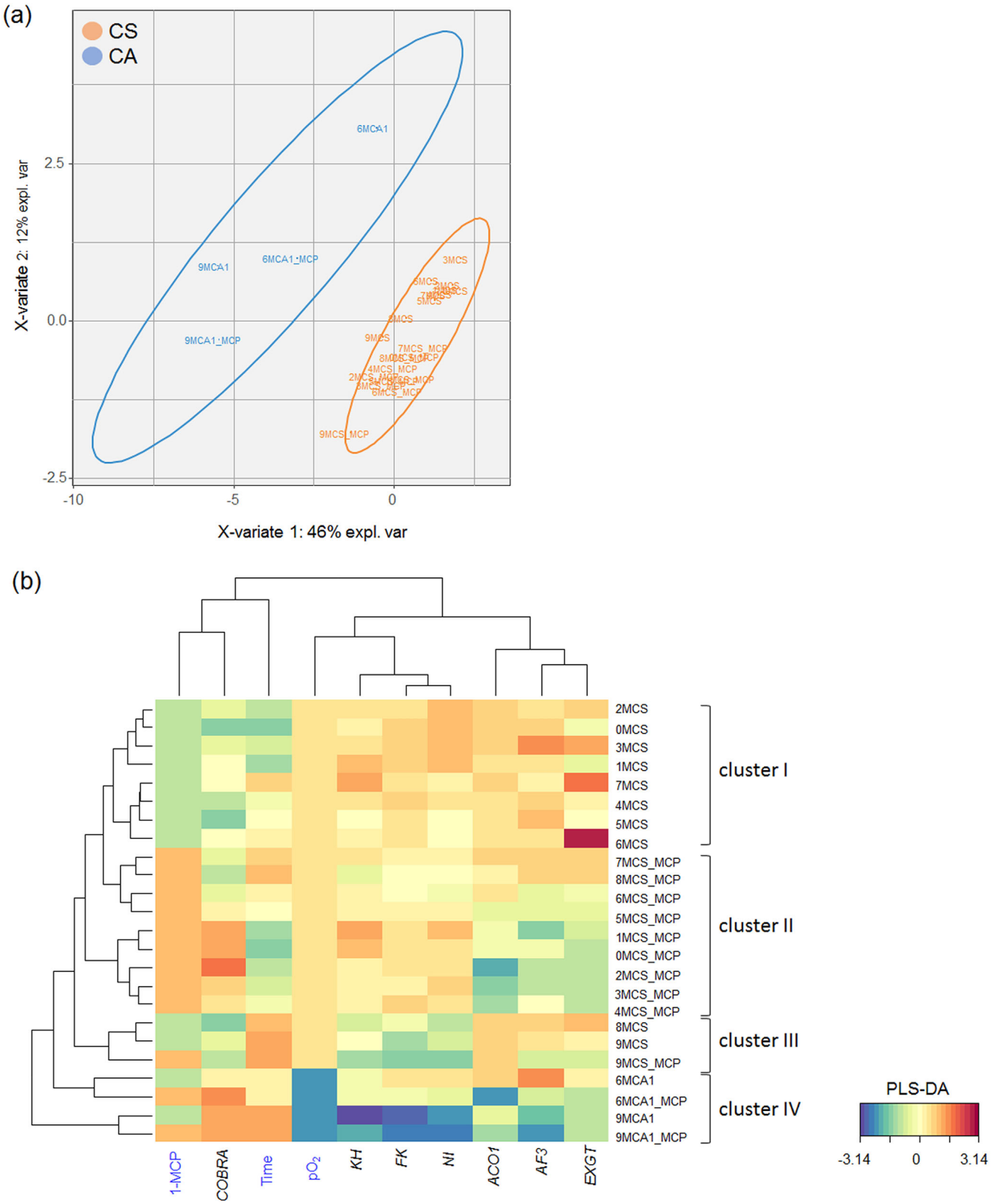

Figure 8. Partial least square discriminant analysis (PLS-DA) model of the expression profile of the investigated genes in fruits kept under CS and CA (**a**). Clustered image map (CIM) of the real-valued PLS similarity matrix between the gene expression profiles, storage conditions, and time under storage (**b**). Rows represent sampling points throughout storage (0 to 9 months) time, under cold- (CS) or controlled-atmosphere (CA) storage, in the absence or presence of 1-MCP treatment (MCP). Simultaneous hierarchical clustering of rows and columns generated CIM. Variables are color coded based on their PLS values, and rows and columns are reordered according to a hierarchical clustering. Gene expression and storage condition variables are in black and blue, respectively.

mealiness (negative) (Fig. 5(b)). The *v*-test of the qualitative supplementary variables RT, CS, and CA for dimensions 1 to 5 resulted in values of −0.01354402, 4.035479, −2.230843, 0.5051255, −0.4622138; 5.58624042, 2.141242, 2.430383, −1.8225622, 0.6820714 and − 5.71742105, −4.211860, −1.374980, 1.6150078, −0.4678082, respectively.

Gene expression profiling in apple during long-term storage

The expression of a group of genes involved in the fruit ripening processes was investigated in apples kept at RT, under CS (1 to 9 months), and CA (6 and 9 months at pO_2 of 1.0 kPa) with and without 1-MCP treatment (Fig. 6). Blockage of ethylene perception by 1-MCP inhibited *MdACO1* transcription up to 5 months under CS and up to 9 months under CA 1.0 kPa (Fig. 6(a), (b)). The reduction of transcription caused by 1-MCP was relieved in later storage periods, from the sixth to the ninth month under CS. A similar regulation pattern was observed for *MdAF3* and *MdEXGT* (Fig. 6(a), (b)).

The expression levels of *MdHK*, *MdFK*, and *MdNI* were low in stored apples, and were shown to be slightly repressed in response to ethylene blockage after longer periods of post-harvest (8 and 9 months under CS, 6 and 9 months under CA) (Fig. 6(a), (b)). Ethylene blockage exerted stronger negative effect on the transcription of *MdHK* and *MdFK* than on *MdNI* (Fig. 6(b)). The inhibition of ethylene perception had opposite effects on the transcription of *MdCOBRA* and *MdSuSy* under RT and in the initial stages of CS, relieving the repression of the first and inducing the transcription of the later (Fig. 6(a), (b)). Under CA pO_2 1.0 kPa, the steady state mRNA levels of both genes increased (Fig. 6(b)).

The hierarchical clustering of gene expression profiles was consistent with metabolic functionality (Fig. 7). The identified clusters demonstrate transcriptional co-regulation of sequences encoding cell-wall associated carbohydrate (cluster I) and hexose metabolism enzymes (cluster II) (Fig. 7). The expression pattern of *ACO1* was similar to those of sequences encoding wall modifying enzymes (Fig. 7). The expression profile of *SuSy* and *COBRA* were more divergent, exhibiting distinct responses to ethylene blockage and time under storage (Fig. 7).

To integrate the transcriptional data to storage conditions and period, we employed partial least square (PLS) discriminant analysis (DA), using the post-harvest technique CS and CA as discriminants (Fig. 8(a)). A large portion of the variance in gene expression (58%) could be explained by storage conditions under CS or CA, and a clear separation between the profiles of the individuals under these conditions was observed (Fig. 8(a)). Relative O_2 pressure had significant loading weights in the first and second components for CS (Fig. 8(b)). In contrast, time had more weight on CA, for the first and second component (Fig. 8(b)). The expression profile of all the genes that were investigated, except for *MdCOBRA*, had more weight on CS than on CA (Fig. 8(b)).

The final gene expression signatures under post-harvest conditions, analyzed by PLS-DA and represented as a clustered image map (CIM) using Euclidean distance and complete linkage, demonstrated four distinct clusters of individuals representing the shorter periods (up to 6 months) under CS (cluster I), CS in the presence of 1-MCP (cluster II) up to 8 months, later CS (cluster III), and CA (cluster IV) (Fig. 8(b)). The relevant interactions between gene expression patterns and storage conditions are also represented as a network supporting information (Fig. S3).

DISCUSSION

Low temperatures and atmosphere manipulation in post-harvest fruit conservation are used to impair the developmental and metabolic processes associated with later ripening and senescence.[18,30] The extended lifespan under storage was accompanied by physiological changes, reflected in fruit physicochemical and sensory properties,[12,18] which influence consumer acceptance negatively.[6,19,20,30] To further understand the effects of long-term storage in apple, we coupled instrumental and sensory analyses to expression profiling of candidate genes involved in late ripening and senescence processes.

Post-harvest technologies delay senescence but do not prevent it completely

The post-harvest technologies that were investigated, including reduced temperatures and atmosphere manipulation via low O_2 pressure and inhibition of ethylene perception, extended apple lifespan but were not able to prevent ripening processes fully. Fruit physicochemical attributes changed with time under storage and global appreciation decreased. Manipulation of the storage atmospheric conditions by applying 1-MCP and reducing O_2 concentration was associated with the retention of desirable attributes, although with smaller effects at the later stages of storage. The effectiveness of atmosphere manipulation in post-harvest conservation technologies is attributed to its ability to reduce and/or suppress the aerobic metabolism in stored fruit directly or indirectly via ethylene-mediated processes.[6,11]

The ability of plants to regenerate novel ethylene binding sites after exposure to 1-MCP and the larger number of transcriptionally active ethylene receptor genes in climacteric fruits[11,31] may be responsible for the failure of the hormone inhibitor in extending conservation after 5 months under CS. The restoration of post-ripening/senescence developmental programs after 5 months under CS in the presence of the ethylene inhibitor is also evidenced by the transcriptional profiles of *MdACO1*, *MdCOBRA*, *MdAF3*, and *MdEXGT*. Similar restoration of ethylene-mediated processes was observed in apples and pears treated with 1-MCP.[32,33]

Under CA, the physicochemical and sensory attributes of 'Gala' apples were mostly retained. Reduced O_2 levels trigger hypoxia adaptation mechanisms in plants,[13] likely responsible for the long-lasting effectiveness of post-harvest technologies based on the control of oxygen relative pressure. Intense transcriptional reprogramming is observed in fruit under low oxygen pressures.[13] Hypoxia also affects directly ethylene biosynthesis and tissue responsiveness to the hormone.[13,14] The metabolic pathways most significantly affected by hypoxia are sucrose catabolism, anaerobic fermentation, and reactive oxygen species regulation, and current evidence indicates that low oxygen levels affect differently ripening and senescence metabolic process.[34,35]

Firmness was the trait exhibiting the most favorable effect of post-harvest technologies, and as shown previously, it is crucial for consumer preference.[6,7,16,18–20] Flesh firmness loss in pomes is associated with ethylene- induced cell-wall degradation.[6,16,19,20] Application of 1-MCP was more effective than hypoxia in retaining flesh firmness in our experimental conditions. The comparatively higher effectiveness of ethylene blockage in preventing firmness loss is likely due to the role of ethylene in triggering abscisic acid (ABA) biosynthesis, which further accelerates cell-wall degradation.[4,5]

Apple acidity is mainly determined by the malic acid content, accounting for up to 90% of its total organic acids. Our results

demonstrated a positive effect of ethylene inhibition on retaining fruit acidity, as observed previously.[7,9] However, senescence-induced loss of malic acid was not fully prevented after 6 months under cold storage and 9 months under controlled atmosphere storage, in the absence of ethylene perception.

The accumulation of soluble solids in apples depends on the genomic context and cultural practices.[36,37] In apple, fruit growth occurs at faster or similar rates than sugar synthesis and accumulation.[36] The energy needed for fruit and seed development is provided by hexoses, arising from the breakdown of photosynthetic sucrose and accumulated in the vacuoles of the cells.[4,5] In long-term storage, the soluble sugar content was not significantly affected by the atmospheric conditions that were investigated or by ethylene inhibition, as shown in previous work.[32] The crucial role of soluble sugars in fruit and seed energy metabolism may contribute to a smaller influence of ethylene, in comparison to that of developmental processes.

Sensory evaluation poorly reflects physicochemical and transcriptional changes

Fruit quality depends strongly on consumer acceptance, which is based mainly on sensory properties, such as appearance, aroma, taste, and texture.[38] Multifactorial analysis of physicochemical and transcriptional changes in apple during storage have shown that, although it is possible to determine the relative contribution of certain variables to sensory properties, such as storage time and ethylene blockage under CS, most of the observed modifications are not perceived in sensory evaluations. Discrepancies between instrumental and sensory evaluations in apple were reported in previous studies.[19,22]

The texture of apple fruits is a complex trait, controlled by several regions in the apple genome.[20,39] The sensory evaluation of mealiness in a segregating population of apple allowed the identification of a novel gene encoding an arabinofuranosidase, whose expression levels correlated with the undesirable texture trait.[20] In our study, the expression profile of *MdAF3* allowed the discrimination between early stage CS and CS with 1-MCP treatment and CA storage. Other genes associated with carbohydrate, cell-wall and ethylene metabolisms also exhibit valid discriminatory potential. The expression profile of an apple orthologue of the extracellular glycosyl-phosphatidyl inositol-anchored protein, COBRA, from *Arabidopsis thaliana*, was also associated with the loss of sensory quality at later stages of storage. In pear, the expression of *COBRA*-like genes was also positively correlated with reduced fruit quality resulting from firmness loss.[33] In contrast, the expression of genes encoding proteins of the hexose metabolism is preferentially regulated by developmental cues in apples under long-term storage. The transcription profiling of the genes that were investigated allowed discrimination between fruits stored under CA and CS, which received similar grades to sensory attributes. Thus, molecular tools may function as predictive tools for senescence in apples during storage.

Inhibition of ethylene perception affects gene expression in stored apples

The impairment of ethylene perception and signaling altered the expression of genes associated with the cell-wall metabolism and hormone biosynthesis (*MdCOBRA, MdAF3*, *MdEXGT*, *MdSuSy*, and *MdACO1*), as shown in previous studies.[15,18] The effect on the regulation of the expression of genes in the hexose metabolic pathway (*MdNI*, *MdHK* and *MdFK*) was less pronounced. In fact, in our experimental conditions, the transcriptional profile of hexose metabolism genes was associated with time and O_2 pressure. As observed for other sink organs, the cleavage of sucrose in apple is initiated by the activity of invertases or sucrose synthases, releasing glucose and fructose or uridine 5′-diphosphoglucose (UDP-G) and fructose, respectively.[40] Glucose is specifically phosphorylated by hexose kinases, whereas fructose can be phosphorylated by hexose or fructokinases. Fructokinases are considered key enzymes in plant carbohydrate metabolism, and are critical to biomass formation in sink tissues.[40] The expression profile of the genes encoding hexose metabolism enzymes in apple during long-term storage is consistent with their role in regulating carbon fluxes in response to endogenous cues and exogenous conditions. These observations indicate that sucrose is synthesized *de novo* from glucose and fructose during storage in apple.

The transcriptional profile of *MdCOBRA* was the most significantly affected by the absence of ethylene perception. The *COBRA* gene family, previously characterized in *Arabidopsis*, tomato and monocotyledonous rice and maize,[41] encodes a plant-specific glycosylphosphatidylinositol (GPI)-anchored protein with a predicted cellulose-binding site.[42] COBRA is anchored to the extracellular side of the plasma membrane via a glycosylphosphatidylinositol (GPI) moiety and functional analyses in *Arabidopsis* and rice demonstrated its involvement in cytoskeleton orientation during cell division.[41,42] In tomato, a *COBRA-like* gene has been associated with the initial stages of fruit development and the control of firmness at later stages.[43] Our results suggest that the apple orthologue investigated is also under developmental control because, in CS, the inhibition of ethylene perception by 1-MCP was not sufficient to repress its transcription to the levels observed at the initial 5 months under storage. A possible role of oxygen in controlling *MdCOBRA* transcription, in an ethylene-independent way, is suggested, because its expression remained low after 9 months in CA.

The role of ethylene in controlling the transcription of the gene encoding the last enzyme in its biosynthesis, aminocyclopropanecarboxylate (ACC) oxidase (ACO1),[44] and of the α-L-arabinofuranosidase *MdAF3*[16,18,20] agrees with previous works in apple. A similar ethylene-dependent transcriptional regulation pattern was observed for *MdEXGT*, a gene coding for a xyloglucan degradation enzyme predominantly acting on cell-wall hemicellulose. Previous studies in apple and persimmon, demonstrated that EXGT activity is involved in ethylene-dependent firmness loss.[45]

Among the genes involved in carbohydrate metabolism, *MdSUSY* exhibited the most marked ethylene-regulated profile. The transcription of *MdSUSY* was increased at the initial stages of CS, from the second to the sixth month, in the presence of 1-MCP and under CA, indicating that, in the absence of the hormone, apples are able to resume a subset of metabolic activities suppressed by ethylene during ripening.[15,18] After 7 months under CS, blockage of ethylene perception repressed *MdSUSY* transcription, suggesting the involvement of developmental factors, as observed in *Arabidopsis*.[46]

Oxygen levels affect transcriptional regulation in stored apples

The combined use of 1-MCP and CA during storage is recommended in apple conservation.[44,47] However, our results indicate that sensory and physicochemical properties are retained effectively under CA in the absence of 1-MCP. The use of 1-MCP reduced deleterious effects of time under CS. Similar results were shown previously for 'Gala' apples under ultra-low oxygen (1 kPa O_2).[48] Other studies have shown that 1-MCP, or its analogues, in combination with CA significantly delayed fruit softening during

long-term storage.[7,15] The conflicting results are likely due to different genomic contexts and pre-harvest conditions.

Reduction in oxygen levels did not qualitatively affect the expression of the investigated genes, although quantitative differences were observed. Reduction in O_2 level markedly influenced the transcription of genes involved in carbohydrate metabolism (*MdSUSY*, *MdNI*, *MdHK*, and *MdFK*). The increased transcription of these genes triggered by senescence was potentiated by the reduction in available O_2 under CA. Low oxygen levels induce severe energy stress in plants and animals, primarily by promoting fermentation and glycolysis.[13,14,34] The upregulation of these genes may reflect the energy imbalance caused by hypoxia. The condition is more significant in tissues with low permeability to oxygen diffusion, such as apple pulp.[13,14] Transcription factors of the APETALA2/ ETHYLENE-RESPONSIVE FACTOR (AP/ERF) family regulate sugar metabolism, fermentation and/or growth in plants under low-oxygen conditions,[34] thus providing a direct link between ethylene-mediated developmental processes and hypoxia. The gene coding for the principal enzyme in ethylene biosynthesis, *MdACO1* was also repressed under low O_2, although it was relieved with time. The expression of the putative transcriptional regulator of pectin hydrolysis *MdCOBRA* was not significantly affected by hypoxia. The observed profile agrees with previous studies in other species that suggest a developmentally controlled role for COBRA.[33,43]

Among the factors contributing to post-harvest conservation of apples, low temperatures, and hypoxia act as cellular stress signals, whereas the blockage of ethylene perception functions in the opposite direction, preventing the cells to respond to the environmental stresses. Moreover, these stress responses are integrated into the larger developmental programs of ripening and senescence. Thus, post-harvest quality preservation depends on the integration of complex physiological and metabolic processes.

CONCLUSION

Our results demonstrated that post-harvest storage conditions, coupled with atmosphere manipulation, effectively delay apple senescence at the molecular level up to 9 months. The reduction in relative oxygen pressure under controlled atmosphere is effective to retain fruit quality in the absence of ethylene inhibition. In contrast, apple quality conservation is limited to 5 months under cold storage, even with 1-MCP application. The expression of candidate genes associated with apple quality traits is distinctly regulated by the storage conditions. Transcription of genes encoding enzymes of the hexose metabolism is preferentially controlled by fruit development, whereas the expression of those associated with cell-wall metabolism and ethylene biosynthesis responds to storage condition and atmosphere manipulation. Gene expression and physicochemical analyses detected changes unperceived by sensory evaluations, suggesting their use as precocious quality markers in apple.

AUTHORS' CONTRIBUTIONS

JID, TTS and CP participated in the design, performed instrumental, sensory and gene expression analyses, performed statistical data analyses, and drafted the manuscript. GRC and BXG contributed to instrumental and sensory analyses. VQ and FBF analyzed the data. CVR and CLG designed the experiments. TTS, CP and VQ drafted the manuscript with contributions from CVR and CLG. All authors collaborated in the construction of the hypothesis. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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