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ORIGINAL ARTICLE

Technical benefit on apple fruit of controlled atmosphere influenced by 1-MCP at molecular levels

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Abstract

The apple is a highly perishable fruit after harvesting and, therefore, several storage technologies have been studied to provide the consumer market with a quality product with a longer shelf life. However, little is known about the apple genome that is submitted to the storage, and even less with the application of ripening inhibitors. Due to these factors, this study sought to elucidate the transcriptional profile of apple cultivate Gala stored in a controlled atmosphere (AC) treated and not treated with 1-methyl cyclopropene (1-MCP). Through the genetic mapping of the apple, applying the microarray technique, it was possible to verify the action of treatments on transcripts related to photosynthesis, carbohydrate metabolism, response to hormonal stimuli, nucleic acid metabolism, reduction of oxidation, regulation of transcription and metabolism of cell wall and lipids. The results showed that the transcriptional profile in the entire genome of the fruit showed significant differences in the relative expression of the gene, this in response to CA in the presence and absence of 1-MCP. It should be noted that the transcription genes involved in the anabolic pathway were only maintained after six months in fruits treated with 1-MCP. The data in this work suggests that the apple in the absence of 1-MCP begins to prepare its metabolism to mature, even during the storage period in AC. Meanwhile, in the presence of the inhibitor, the transcriptional profile of the fruit is similar to that at the time of harvest. It was also found that a set of genes that code for ethylene receptors, auxin homeostasis, MADS Box, and NAC transcription factors may be involved in the regulation of post-harvest ripening after storage and in the absence of 1-MCP.

Camila Francine Paes Nunes and Isadora Rubin de Oliveira contributed equally to this work.

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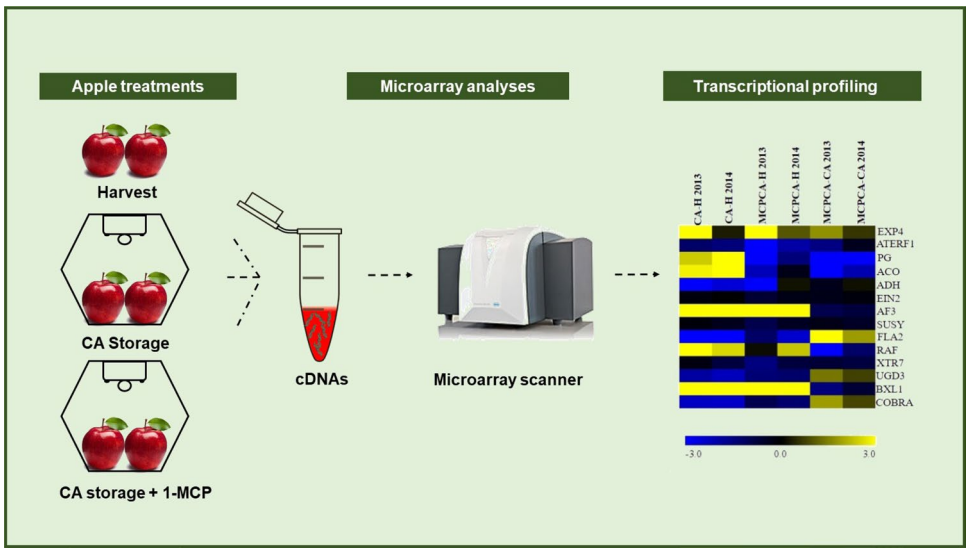
Graphic abstract



Keyword *Malus* × *domestica* · Fruit · Ethylene · Ripening · Maturation

Abbreviations

H	Harvest
CA	Controlled atmosphere
MCP	1-Methylcyclopropene
TA	Titratable acidity
SSC	Soluble solids contents
RT	Room temperature
Cy3	Cyanine-3
Cy5	Cyanine-5
BH	Benjamini-Hochberg
GDR	Genome database for Rosaceae
FDR	False discovery rate
GO	Gene ontology
RT-qPCR	Reverse transcription quantitative Polymerase Chain Reaction
DET	Differentially expressed transcripts
CDs	Coding sequences

Introduction

The apple (*Malus domestica*) is a climacteric fruit highly produced and commercialized in the world (Storch et al. 2015). After harvest, the fruit is usually treated with 1-MCP and stored in a controlled atmosphere (CA) to prolong its commercial useful life (DeEll et al. 2016). However, studies of chemical and physiological responses have shown that CA alone has a great effect on maintaining the quality attributes of the apple, especially the firmness of the pulp, suggesting that the use of 1-MCP is irrelevant (Both et al. 2014; Stanich et al. 2014). Nevertheless, relatively little information is available on metabolic changes during CA and the endogenous regulatory processes that control ripening, ripening, and fruit quality at the genomic level in CA in the presence or absence of 1-MCP is not well understood.

Exploratory studies with some specific genes of apple metabolism have allowed a small advance in the compartmentalized understanding of fruit ripening during and after storage (Cao et al. 2012; Gwanpua et al. 2014; Storch et al. 2015, 2017; Tadiello et al. 2016). Among these researches we have the study of carbohydrate metabolism as the most studied and having as elucidated that ripening and maturation are accompanied by fruit softening resulting from catabolic activities related to cell wall solubilization and degradation by cell wall loosening expansins. and numerous cell wall hydrolases, such as polygalacturonases (PG), pectin methylesterases (PME), pectate lyases (PL), α-L-arabinofuranosidases (AFase), and β-galactosidases (βGal) (Storch et al. 2015; Zhang et al. 2017). Expression of genes encoding cell wall-modifying enzymes in apple is partially by ethylene (Storch et al. 2015). Studies have proposed the existence of active anabolic metabolism occurring post-harvest, including photosynthesis and cell wall biosynthesis (Cao et al. 2012; Tadiello et al. 2016; Storch et al. 2017).

More robust analyzes such as microarray have been used to investigate gene transcription profiles in apples during post-harvest storage (Tadiello et al. 2016; Storch et al. 2017). This approach has allowed us to observe an important role of 1-MCP in the activation of the transcription of genes of anabolic pathways (Storch et al. 2017). However, little is known

about what happens in the other metabolic compartments of the apple during storage. It is necessary to carry out studies with a transcriptomic vision on a large scale that seeks to understand as a whole the events that promote the ripening of the fruit stored in CA in the presence and absence of 1-MCP, identifying key genes for future studies of genetic improvement of the apple tree.

Materials and methods

Plant material and post-harvest treatments

Apples (*Malus* × *domestica* Borkh.) of the 'Gala' variety, clone Baigent, were harvested from experimental orchards in Brazil, in two consecutive harvesting seasons (2013 and 2014). Fruit was divided into two groups (100 kg each) for post-harvest treatments. Each group was divided into three experimental units and either treated with 1 ppm (1 μL L^{-1}) 1-MCP (Smart Fresh™) for 24 h at 0 ± 0.5 °C, or maintained under the same conditions without 1-MCP application. Fruit (both treated and not treated with 1-MCP) were then stored for 6 months under CA conditions (1% O_2; 2% CO_2; 25 ± 0.5 °C and 90% ± 5% of relative humidity), followed by storage at room temperature (RT, 25 °C and 95% of relative humidity) for 7 days after CA. Samples were collected at harvest and after six months under CA storage + 7 days at RT (with and without 1-MCP), submitted to technological analyses, and then frozen in liquid N_2 and stored at − 80 °C until the RNA extraction and microarray analyses were performed.

Technological analyses

Samples were characterized for flesh firmness (FF), soluble solid content (SSC) and titratable acidity (TA). All measurements were performed as described by Storch et al. (2017). Statistical analyses, viz., ANOVA and Tukey's Test, were performed using package Agricolae in R (de Mendiburu 2016).

RNA extraction and transcriptional profile

Total RNA was extracted following the protocol described by Zeng and Yang (2002) and modified according to Storch et al. (2015), from 6 g of frozen flesh material obtained from a pool of five representative fruit, at each sampling point, from both growing seasons. Total RNA was quantified spectrophotometrically using NanoDrop ND-1000 (NanoDrop Technologies, USA), and its integrity was confirmed by agarose gel electrophoresis absence of DNA was confirmed by PCR. Preparation of amplified RNA (aRNA), cDNA transcription, and hybridization targets co-hybridized to AryANE 12× 135 K microarrays (Celton et al. 2014). NimbleGen microarray AryANE v1.0 containing 135,000 60-mers oligonucleotide probes.

Hybridizations were conducted overnight in a NimbleGen Hybridization System 4 (Roche NimbleGen, USA) (mix mode B) at 42 °C. Subsequently, the slides were washed, dried, and scanned at 2 μm resolution. Fluorescence data were extracted from the scanned images using NimbleScan Software v2.4. Treatments—Harvest (H), CA storage + 7 days RT (CA), and 1-MCP treatment combined with CA storage + 7 days RT (MCPCA)—were compared in three paired combinations (CA-H; MCPCA-H; MCPCA-CA). Each comparison was hybridized into two microarrays, one for each harvesting season (2013 and 2014), giving a total of twelve microarrays to be analyzed in a dye-swap design. Probe annotation was performed as described by Celton et al. (2014).

Microarray data analyses

Statistical analyses were conducted using software R (R Development Core Team 2014). Fluorescence intensity data were normalized using the Lowess method, and the differential expression analyses employed lmFit and Bayes moderated *t* test functions using LIMMA package (Ritchie et al. 2015), at the Bioconductor project (R Development Core Team 2014). After normalization and significance analysis, the normalized intensity values were subtracted from the background to provide an estimate of transcript expression levels, as described by Celton et al. (2014). Genes that exhibited same behavior during the two growing seasons were considered significantly differentially expressed based on the false discovery rate (FDR) *p* values ($p < 0.01$) after the Benjamini-Hochberg (BH) correction (< 0.05). Differentially expressed sequences were classified in gene ontology (GO) and metabolic pathways, according to MapMan scheme v.3.6.0 RC1 (Thimm et al. 2004). GO enrichment analyses were performed by a hypergeometric test, with a 0.05 significance threshold after BH corrected FDR *p* values, using agriGO (Du et al. 2010), and summarized on the basis of measures of semantic similarity using REVIGO (Supek et al. 2011). To perform further functional analyses, the differentially expressed transcripts from AryANE 12× 135 K chip (Celton et al. 2014), were validated against the *Arabidopsis* genome in TAIR (The Arabidopsis Information Resource–https://www.arabidopsis.org/).

Relative transcripts accumulation by RT-qPCR

Microarray expression data were validated by RT-qPCR for fourteen genes chosen on the basis of their expression profiles and putative biological functions. Primers were designed based on the coding sequences (CDs) of

Malus × *domestica* genome available at GDR (Genome Database for Rosaceae—https://www.rosaceae.org/), using the software Primer3Plus (Untergasser et al. 2007), to generate amplicons ranging from 80 to 150 base pairs (Supplementary Table S1). cDNA synthesis and qPCR were performed as described by Storch et al. (2015). Results were normalized using reference genes *Porin* (MDP0000271281), *Histone* (MDP0000223691), and *Drought-Responsive Family Protein* (MDP0000217860), which were chosen based on their stable transcriptional profile in the microarray analysis and the Datassist and geNorm algorithms' indication by Storch et al. (2015). The expression profile validation for the fourteen genes is presented in Supplementary Figure S2.

Results and discussion

Fruit technological characterization

Apples were harvested with the same flesh firmness and soluble solids content in both growing seasons (Fig. 1a, b), however, acidity was lower in apples harvested in 2014 when compared to 2013 (Fig. 1c). Fruit firmness remained unaltered during storage regardless of 1-MCP treatment (Fig. 1a). The apples at the end of storage regardless of 1-MCP treatment had higher soluble solids content when compared to apples at harvest (Fig. 1b). When comparing cold-stored apples, 1-MCP treatment only influenced soluble solids content in the 2013 growing season when treated apples (MCPCA) had lower soluble solids content than those stored without 1-MCP treatment (Fig. 1b).

Acidity was reduced upon storage in both growing seasons regardless of 1-MCP treatment. Cold-stored apples had lower acidity in 2014 than cold-stored apples in 2013 (Fig. 1c). Taken together, these results demonstrate that 1-MCP and CA storage impact mainly the apple flesh firmness maintenance and do not stop SSC and TA changes, which is in agreement with the previous works that have demonstrated high ethylene-dependence of flesh firmness (Storch et al. 2015, 2017). The 1-MCP and CA combination have been shown to effectively delay a reduction in TA and SSC in 'Gala', 'Delicious' and 'Granny Smith' apples (Bai et al. 2005). The authors Storch et al. (2017) demonstrated that environmental conditions are the main factors affecting the physiological traits, including transcriptional profiles in apple fruit.

Apple transcriptional profile and GO enrichment

A large portion of the differentially regulated genes was affected by the growing season with the transcriptional pattern profile according to the year, mainly in the transcript related to lipid metabolism. These analyzes only the effect of treatment, a total of 2856 genes exhibited significant differential regulation. The highest number of transcripts was observed in the CA-H comparison (1952). In the MCPCA-H and MCPCA-CA comparisons, a lower number of transcripts were observed (527 and 377, respectively). Significant

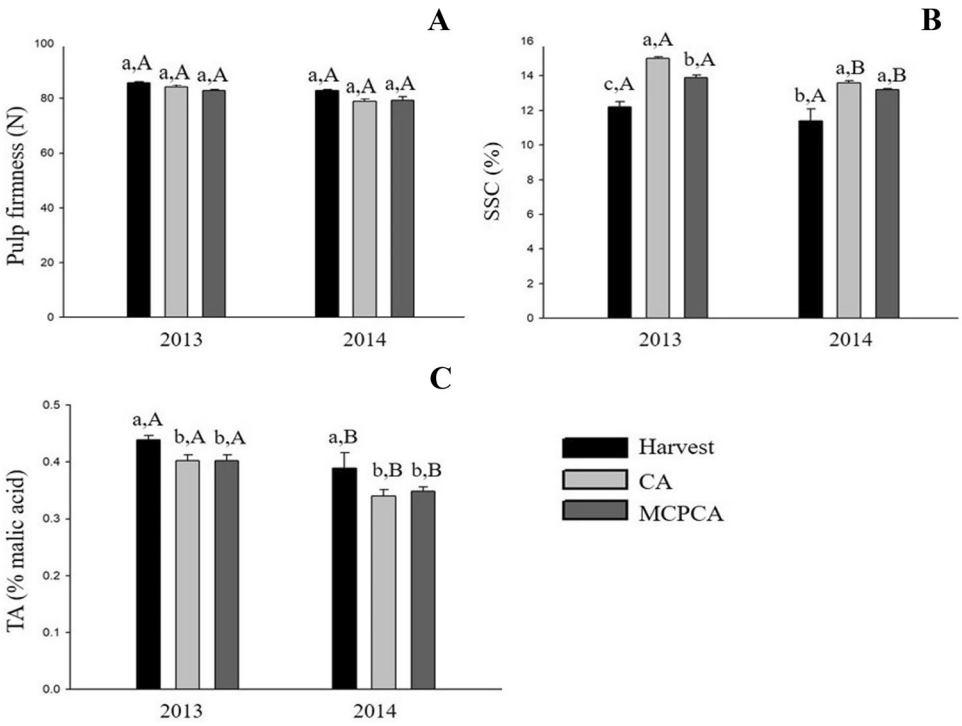


Fig. 1 Flesh firmness, soluble solids content (SSC), and titratable acidity (TA) of 'Gala' apples after harvest, six months under the controlled atmosphere (CA—1% O_2, 2% CO_2, 0 ± 0.5 °C, 90% relative humidity ± 5%) with (MCPCA) and without the application of 1-methylcyclopropene (CA) (results are means of two experiments occurring in two consecutive years). Means of treatments within a year followed by different lower-case letters are significantly different and means of treatments compared across years followed by different capital letters are significantly different by Tukey's test ($p < 0.05$). A significant interaction between year and treatments was observed for all variables (ANOVA $p < 0.01$)

enrichment classification demonstrated by Gene Ontology (GO) was observed for various processes related to catabolic and anabolic pathways (Fig. 2a–c).

The GO classification of the genes under biological processes demonstrated significant enrichment in the processes related to photosynthesis, carbohydrate metabolism, response to a hormonal stimulus, nucleic acid metabolism, oxidation–reduction, transcription regulation, and cell wall and lipid metabolism (Fig. 2a–c). The most frequent cellular component ontology terms identified in the three comparisons were Golgi-associated vesicle membranes, vesicle membrane, photosynthetic membrane, and thylakoid (Supplementary Figure S1). Based on GO enrichment results, the transcripts related to the cell wall, photosynthesis light reactions, lipid, hormonal, and transcription factors' metabolic pathways were selected to carry out the discussion.

To validate the microarray transcriptional profiles, fourteen transcripts were further investigated by RT-qPCR (Supplementary Table S1). In general, the transcript behavior

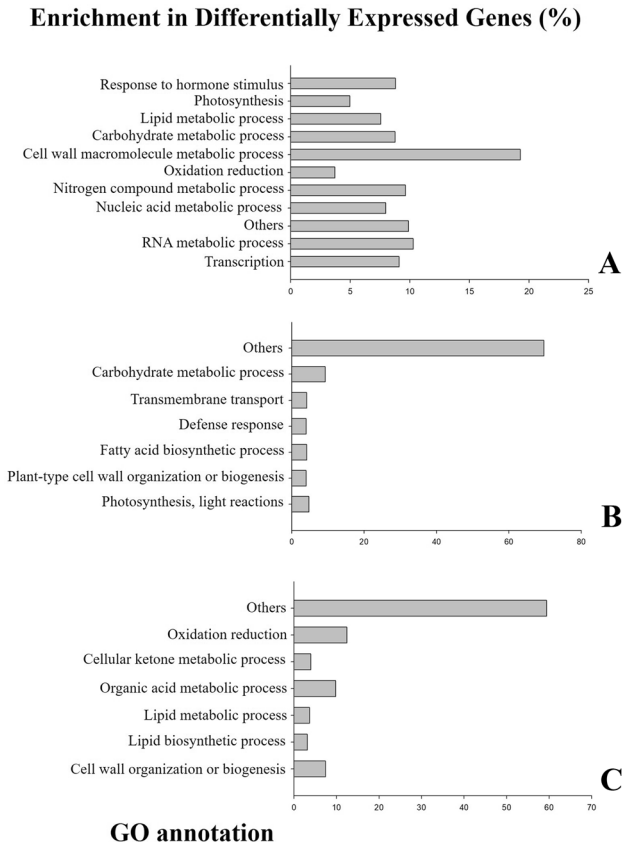


Fig. 2 Enrichment in gene ontology classifications. The analysis was performed for the comparisons: CA storage versus harvest (CA-H) (**a**), CA storage in the presence of 1-MCP versus harvest (MCPCA-H) (**b**), and CA storage in the presence of 1-MCP versus CA storage (MCPCA × CA) (**c**). Gene ontology categories exhibiting low significance enrichment, according to *p* value and FDR, were grouped into the category "others"

profile was found to be similar regardless of the technique used for the two harvesting seasons for 78.6% of the genes (Supplementary Figure S2).

The 1-MCP maintained the transcripts for cell wall biosynthesis under CA storage

Fruit softening has been reported to be a result of changes in the cell wall solubilization, leading to a reduction in the intercellular adhesion, pectin depolymerization and solubilization, hemicelluloses depolymerization, and loss of galactose, arabinose and xylose from the side chains (Brahem et al. 2017). It has been reported that the decrease in flesh firmness is accompanied by an increase in the gene expression and activity of the cell wall degrading enzymes, such as polygalacturonases (PG), pectin methylesterases (PME), *β*-galactosidases, *α*-L-arabinofuranosidases, *β*-xylosidases, and expansins (Gwanpua et al. 2014; Storch et al. 2015). However, the association between the increase or maintenance of mRNA content-coding for cell wall biosynthesis enzymes and the maintenance of fruit flesh firmness has not been reported. A large number of transcripts encoding cell wall degrading enzymes were down-regulated in cold-stored apples when compared to apples at harvest (CA-H) (Fig. 3; Supplementary Table S2 and S3).

Meanwhile, cold stored apples treated with 1-MCP had similar transcription profiles of certain genes related to cell wall biosynthesis when compared to fruit at harvest (MCPCA-H; Fig. 3; Supplementary Table S2 and S3). This difference in the transcriptional profiles did not result in differences in the apple flesh firmness. Cold-storage alone was sufficient to maintain apple flesh firmness. Post-transcriptional and translational events may be involved in the regulation of quality traits. Authors have also suggested that gene expression and quality traits (such as flesh firmness, color change and odor) have different patterns of regulation and ethylene dependence (Ireland et al. 2014). Transcripts encoding cell wall biosynthesis enzymes such as *UGD3* (MDP0000193220, MDP0000146452, MDP0000780281, MDP0000132475), *GAE3* (MDP0000925736, MDP0000780281, MDP000164430), *GAE6* (MDP0000216173), *FLA2* (MDP0000282305), *FLA17* (MDP0000156830), and *Rhamnose biosynthesis 3* (MDP0000179744), were observed to be unregulated in stored apples treated with MCP when compared to non-treated stored apples (MCPCA-CA; Fig. 3; Supplementary Table S2).

The *UGD3* encodes a UDP-glucose 3-dehydrogenase that produces UDP-D-glucuronic acid (UDP-D-GlcA) from UDP-D-glucose (UDP-D-GLc) (Reboul et al. 2011). UDP-D-GlcA is a common precursor for arabinose, xylose, galacturonic acid, and apiose residues found in the fruit cell wall (Reboul et al. 2011). The UDP-glucose 3-dehydrogenase competes

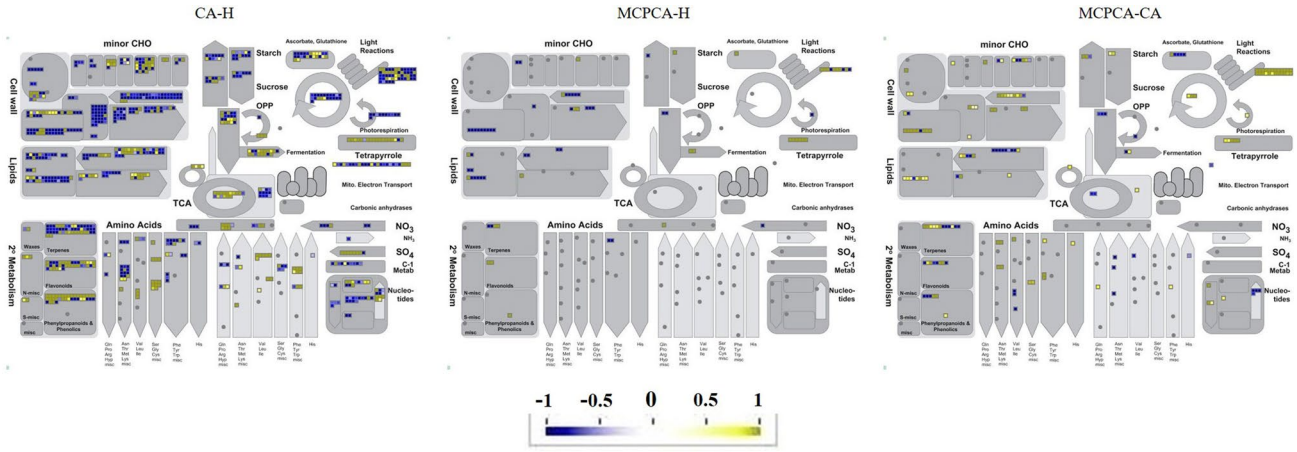


Fig. 3 Graphical representation of the metabolism overview distribution of the differentially expressed transcript, according to the MapMan scheme. The comparisons correspond to CA storage versus harvest (CA-H), CA storage in the presence of 1-MCP versus harvest (MCPCA-H), and CA storage in the presence of 1-MCP versus CA storage (MCPCA-CA). Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Up-regulated genes are shown in yellow and down-regulated ones in blue according to the color scale

directly with sucrose-6-phosphate synthase enzyme for UDP-D-GLc substrate released by sucrose synthase (SUS) in the recycle of sucrose, affecting the direction of a metabolic pathway for simple sugars (Fig. 4). This metabolic pathway, which redirects the simple sugars to cell wall-carbohydrate synthesis, limits the soluble sugars content (Reboul et al. 2011), which could explain the lower SSC in MCPCA than in CA (Fig. 1).

The *GAE3* and *GAE6*, which encode enzymes that produce D-galacturonic acid (Reboul et al. 2011) (Fig. 4). The D-galacturonic acid constitutes pectin in the cell wall through α–1–4 linkage with homogalacturonan in rhamnogalacturonan-I and is also found in the side chains of rhamnogalacturonan-II polysaccharide complex, which is involved in cell wall cross-linking with boron (O'Neill et al. 1996).

An up-regulation of cellulose synthesis genes (MDP0000279573, MDP0000847392, MDP0000263817), and FLA proteins (MDP0000282305, MDP0000156830), in the MCPCA-CA comparison was observed. The FLA encodes glycoproteins that contain glycosylation sites for bonding arabinogalactan and arabinose polysaccharides into the cell wall matrix (Johnson et al. 2003). The mutants

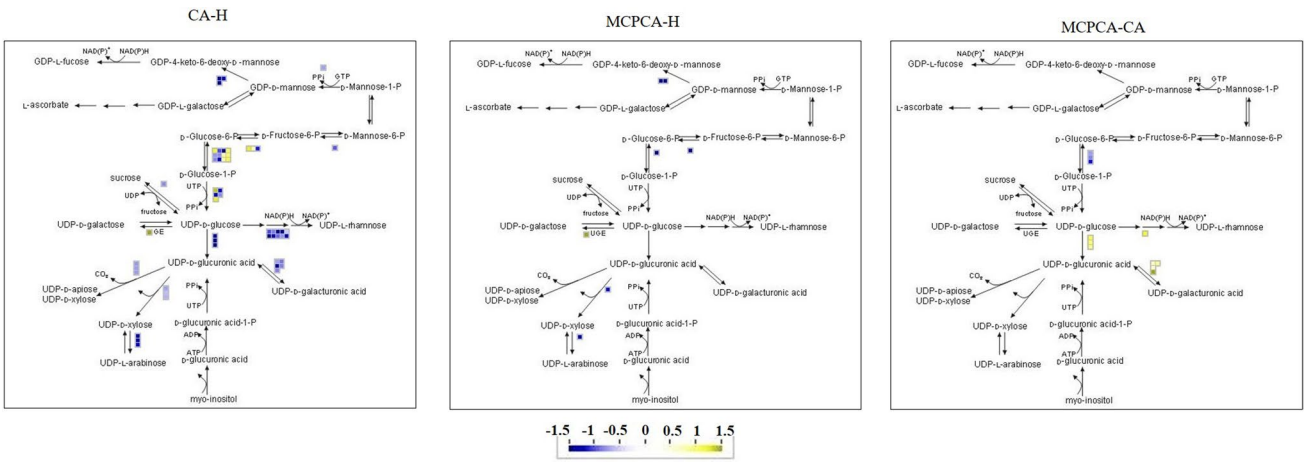


Fig. 4 Graphical representation of cell wall precursor distribution of the differentially expressed transcript, according to the MapMan scheme. The comparisons correspond to CA storage versus harvest (CA-H), CA storage in the presence of 1-MCP versus harvest (MCPCA-H), and CA storage in the presence of 1-MCP versus CA storage (MCPCA-CA). Gray boxes represent metabolic pathways and colored squares represent the genes, according to the transcriptional fold change in each given comparison. Upregulated genes are shown in yellow and downregulated ones in blue according to the color scale

of *Arabidopsis* with suppressed *FLA11* (AT5G03170) and *FLA12* (AT5G60490) genes have been reported to exhibit stems with lower tensile strength and elasticity, as well as an increase in the angle of cellulose microfibrils accompanied by a decrease in arabinose, galactose, and cellulose (Macmillan et al. 2010). Furthermore, these transcripts for FLA11 and FLA12 were highly correlated to cellulose synthesis in *Arabidopsis* (Macmillan et al. 2010). Authors have also suggested that the FLAs proteins are secondary cell wall components, with the specialized function of adhesion among the cellulose microfibrils (Macmillan et al. 2010). According to a hypothesis by Qi et al. (2017), the down-regulation of *FLA* genes during the apple ripening promotes the loosening of cell wall adhesions.

The *RHM1* (MDP0000179744) [a transcript that encodes the enzyme for biosynthesis of L-Rhamnose (Rha), which is an important constituent of fruit's pectin cell wall (Wang et al. 2009)] and *RGXT2* (MDP0000150593) [transcript encoding a xylosyltransferase involved in the pectic–rhamnogalacturonan-II biosynthesis (Egelund et al. 2006)] were also unregulated in 1-MCP treated apples when compared to CA apples, maintaining the transcription levels after 6 months as they were at the time of harvest. Therefore, we have observed that while the CA technology is adequate to decrease the transcription of genes coding for cell wall-degrading enzymes, its association with 1-MCP results in the maintenance of transcription of the genes coding for enzymes related to cell wall synthesis. This finding agrees with the recent works published by Busatto et al. (2017) and Storch et al. (2017), which demonstrated that 1-MCP application in apple fruit at RT and CS, respectively, induced de-repression of the genes that are usually expressed in the juvenile stages of development and not in the full-ripening phase.

In our work, most of the genes related to the cell wall biosynthesis up-regulated by 1-MCP are associated with the side chains of pectin, which are rich in neutral sugars such as arabinose, galactose, and xylose. The pronounced loss of arabinose in the post-harvest ripening has already been reported to be related to fruit softening (Brahem et al. 2017). Therefore, we hypothesize that the loss of neutral sugars from the pectin polysaccharides during the post-harvest period of apples may involve, besides the action of degrading hydrolases, at least in part, the repression of genes and the consequential lack of activity of the cell wall biosynthesis enzymes.

The 1-MCP maintained the transcripts for photosynthesis light reactions under CA storage

The transcription profile of genes related to photosynthesis light reactions exhibited a behavior similar to the transcripts for cell wall biosynthesis. We could observe a maintenance effect on the photosynthesis transcripts by 1-MCP under CA storage. In the MCPCA-CA comparison, transcripts coding for the main groups of light-harvesting complex and photosystem's subunits exhibited an over-expression (Fig. 3; Supplementary Table S4). On the other hand, in CA-H comparison, transcripts to photosynthesis light reactions were down-regulated and no difference in the expression was observed when compared to MCPCA-H comparison (Fig. 3; Supplementary Table S4). Therefore, the effect of 1-MCP on photosynthesis transcripts is mainly of maintenance and not of induction, as observed with the transcripts to cell wall biosynthesis as well.

Chlorophyll *a* and *b* are linked together in the supercomplexes formed in photosystem *I* and *II* (LHCI and LHCII) and are responsible for the reception of light energy in the photochemical stage of photosynthesis (Rochaix 2014). In the fruit ripening and maturation process, the occurrence of chlorophyll degradation has been reported (Peng et al. 2013), and in some fruit, this process is ethylene-dependent (Peng et al. 2013), or partially ethylene-dependent or independent (Gonçalves et al. 2013). Sakuraba et al. (2009) suggested that chlorophyll degradation promotes destabilization and degradation of LHC supercomplexes.

The transcripts coding for LHCs proteins has also been reported to be responsive to abiotic stress conditions, which can lead to photoinhibition of the photosynthetic machinery through an increased production of reactive oxygen species (ROS) (Gurunani et al. 2015). NPQ (non-photochemical quenching) is a major photoprotective response to overcome this issue, and PSBO protein, a subunit of the photosystem, contributes to enhancing the photoinhibition tolerance (Yamamoto et al. 2008). The transcripts of *PSBO* (MDP0000858039, MDP0000624964) and *NPQ4* (MDP000028966) were also maintained by 1-MCP under CA storage, suggesting a protective role against oxidative stress. These findings contribute to consolidating the knowledge that variations in the transcript levels of light-harvesting genes can serve as suitable markers for fruit changes during post-harvest storage. Therefore, we suggest that an up-regulation or down-regulation of *LHC* transcripts can serve as a marker for delayed fruit ripening or the beginning of maturation, respectively.

Lipid transcriptional profile in response to 1-MCP and CA storage

The effects of post-harvest technologies for fruit conservation on the transcripts for lipid metabolism are associated with the ability of these methods to regulate membrane stability through delayed changes in the glycerolipids, phospholipids, and polyunsaturated fatty acids in thylakoids and others membranes, consequently preventing the loss of fluidity, delaying maturation (Duan et al. 2013). The genes related to

lipid metabolism exhibited different profiles (Fig. 5; Supplementary Table S5). We observed three main groups of genes with different responses to the treatments: genes related to fatty acid biosynthesis, genes related to phospholipid biosynthesis, and the genes related to sphingolipid biosynthesis. Most of the genes belonging to the first two groups did not exhibit a coherent pattern of transcription between the harvest seasons in the MCPCA-CA comparison. On the other hand, the third group presented various genes that exhibited the maintenance of transcription in response to 1-MCP (Fig. 5).

The highest number of transcripts belonging to lipid metabolism that were differentially expressed was observed in the CA-H comparison, with most of them exhibiting down-regulation (Fig. 5; Supplementary Table S5). On the other hand, in the presence of 1-MCP in the comparisons (MCPCA-H; MCPCA-CA), the transcripts to lipid metabolism exhibited a different behavior that was dependent on the year of harvest (Fig. 5). When considering only those lipid transcripts for the MCPCA-H and MCPCA-CA comparisons that exhibited the same profile during the two growing seasons, the majority did not exhibit differential expression (Fig. 5).

Transcripts to phospholipid biosynthesis, such as *NMT1* (MDP0000308907), *PGPS1* (MDP0000134379), *LPAT4* (MDP0000119464), *LPAT5* (MDP0000784365), *DGK1* (MDP0000276007), *PECT1* (MDP0000222486), and *DGD1* (MDP0000443176) were down- regulated in the CA-H comparison, while *phosphatidyl serine synthase* (MDP0000819113), *ATCK1* (MDP0000269604), *SQD1* (MDP0000128286), *FQR1* (MDP0000509613), and *quinone reductase* (MDP0000829416) were up-regulated (Fig. 5).

A *LPAT4* (MDP0000119464) and *LPAT5* (MDP0000784365) encode enzymes for diacylglycerol biosynthesis process (Tasseva et al. 2004), and *DGD1* (MDP0000443176) encodes the enzyme responsible for final assembly of galactolipids in thylakoid membranes, providing stability to photosystem I complex (Kelly et al. 2016). The thylakoid lipids are known to stabilize the protein complexes of the photosynthetic machinery and their composition is regulated in response to environmental changes, besides that, thylakoid lipid environment of the LHCs complexes plays a significant role in determining the structural flexibility of these macro-aggregates and as a result, in the chlorophyll fluorescence (Garab 2014). The decrease in chlorophyll fluorescence is observed when the membrane changes from a fluid to a rigid state and these changes are mainly reported during temperature adaptation, physiological disorders, and maturation process (Tovuu et al. 2013; Lin et al. 2016). Here, we could observe that certain transcripts for phospholipid biosynthesis for thylakoid membranes were down-regulated in CA-H comparison, similar to the transcripts to photosynthesis light reactions. Although 1-MCP had a maintenance effect on the *LHCs* transcripts, the effect of this ethylene inhibitor on transcripts related to phospholipid biosynthesis was dependent on environmental conditions, as no coherent pattern of transcription was observed between the two harvest seasons that were evaluated. Therefore, CA technology was not able to prevent the loss of transcription of genes related to thylakoid membranes' fluidity, and

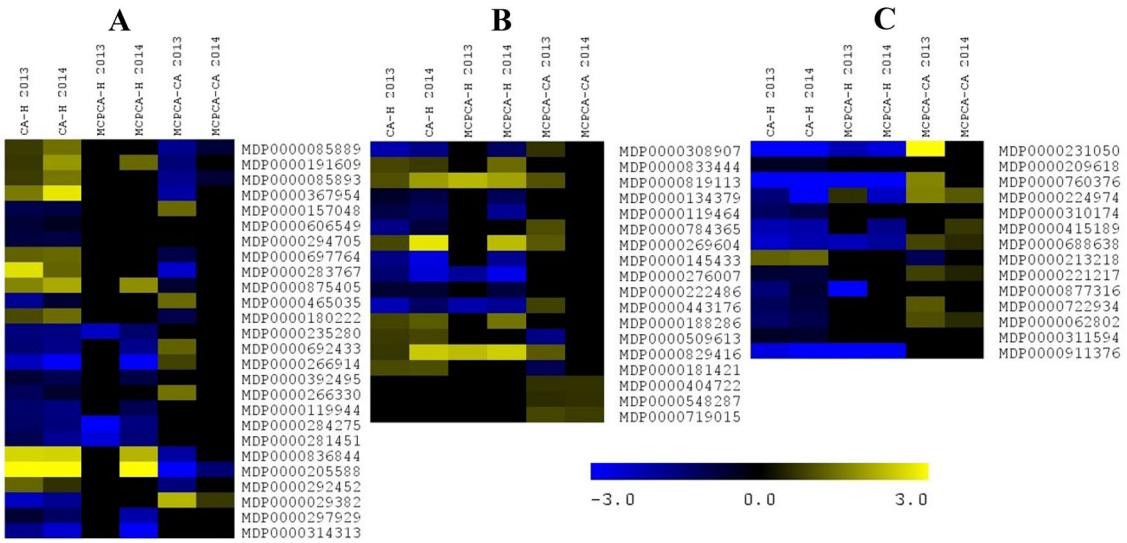


Fig. 5 Transcripts related to lipid metabolism. **a** Fatty acids biosynthetic pathway, **b** phospholipids biosynthesis and **c** Sphingolipids biosynthesis. The transcripts are represented in Multi Experiment Viewer software (TIGR MeV). Blue color represents the minimum expression level and yellow color represents the maximum transcription level observed according to the color scale

it was not possible to hypothesize the role of 1-MCP in this process.

The transcripts about the phospholipid biosynthesis that exhibited upregulation in CA-H comparison were as follows: *phosphatidylserine synthase* (MDP0000819113), which encodes an enzyme for phosphatidylserine biosynthesis process (Michaud et al. 2017), and *ATCK1* (MDP0000269604), which encodes an enzyme for phosphatidylethanolamine (PE) and phosphatidylcholine (PC) biosynthesis (Michaud et al. 2017). The *SQD1* (MDP0000128286), *FQR1* (MDP0000509613) and *quinone reductase* (MDP0000829416) were also upregulated in CA-H comparison. *SQD1* encodes a protein required for sulfolipid biosynthesis [sulfolipid is a lipid present in the photosynthetic membranes] (Michaud et al. 2017). *FQR1* and *quinone reductase* encode enzymes that protect membranes from oxidative stress (Laskowski et al. 2006). Furthermore, *FQR1,* along with SAUR, Aux/IAA, and GH3 families, together form the group of primary auxin-response genes that have been identified (Laskowski et al. 2006); the authors hypothesized that *FQR1* acts in membrane detoxification against auxin-induced oxidative stress. Therefore, it appears that the environment of CA technology prevents oxidative stress since genes related to protection against oxidative processes in the membranes were up-regulated under CA.

Fatty acid desaturases (FADs) are the major enzymes responsible for the synthesis of 18:3, 18:2, 16:3, and 16:2 fatty acids from phospholipids, galactolipids, sulfolipids, and phosphatidylglycerol, in the endoplasmic reticulum (ER) and chloroplast, using cytochrome b5 and ferredoxin as electron donors (Menard et al. 2017). Mutations in the genes coding for this group of enzymes suppress fatty acids polyunsaturation in fruit membrane lipids, a mechanism that is significant for adaptation to temperature changes (Menard et al. 2017). Here, transcripts to *FAD2* (MDP0000284275, MDP0000281451) and *FAD6* (MDP0000029382), which encode enzymes responsible for the synthesis of 18:2 and 16:2 fatty acids, respectively, were down-regulated in CA-H comparison; whereas *FAD3* (MDP0000292452) and *FAD7* (MDP0000836844, MDP0000205588), which are responsible for the synthesis of 18:3 and 16:3 fatty acids, were up-regulated (Fig. 5). On the other hand, in MCPCA-CA comparison, the transcript behavior was reversed; *FAD6* and *FAD7* were up-regulated and down-regulated, respectively. The highly unsaturated fatty acids—18:3 and 16:3—are present abundantly in the thylakoids and in monogalactosyldiacylglycerol, which is the most abundant chloroplast lipid (Vijayan and Browse 2002). Tovuu et al. (2013), observed that the suppression of *FAD7* and *FAD8* in *Arabidopsis* leads to a significant decrease in chlorophyll fluorescence compared to the wild-type.

Sphingolipids are critical components of plant cell membranes and one of the few complex lipids whose disrupted synthesis results in plant lethality (Dietrich et al. 2008). This class of lipids is present ubiquitously in the eukaryotic cells and is involved in various cellular processes, such as signal transduction, protein transport, and programmed cell death; these lipids also form structural components of the plasma membrane and tonoplast (Pata et al. 2010). Here, most of the transcripts to sphingolipid biosynthesis (MDP0000231050, MDP0000209618, MDP0000760376, MDP0000224974, MDP0000310174, MDP0000415189, MDP0000688638, MDP0000221217, MDP0000877316, MDP0000722934, MDP0000062802, MDP0000311594, MDP0000911376) were down-regulated in CA-H comparison and up-regulated in the MCPCA-CA comparison; whereas, in the MCPCA-H comparison, no differential expression was observed (Fig. 5).

This set of microarray results suggest that apples after six months under CA storage in the absence of 1-MCP may begin to exhibit the disruption of thylakoid lipid membranes and LHCs' lamellar structure, as well as a decrease in sphingolipid biosynthesis. These responses can lead to the beginning of maturation processes much before then in the apples stored in CA in the presence of 1-MCP.

Hormonal interplay with transcription factors (TFs) in apples under CA storage

It is well documented that ripening, maturation and maturation processes in climacteric fruit are triggered by ethylene (Seymour et al. 2013). However, advances in research have made it possible to demonstrate that even for climacteric fruit, there is a complex signaling network in which ethylene does not act independently (Trainotti et al. 2007). Some studies have been suggested that ethylene-dependent and ethylene-independent events operate in tandem to regulate the overall ripening and that these events appear to occur in stages before the ethylene burst, along with hormonal interplay and TFs' regulatory circuits (Wu et al. 2016).

The transcripts for the biosynthesis of ethylene, mainly those encode for ethylene receptors were up-regulated in CA-H comparison (Supplementary Table S6 and S7), with an exception of *ACO1* (MDP0000139203). These results suggest that the basal production of ethylene might be occurring even under CA storage. Interestingly, it was also observed that transcripts for homeostasis and response to auxin stimulus were up-regulated in CA-H comparison (Supplementary Table S6). Certain transcripts to the jasmonate pathway (*JAM2*, *LOX1*, *LOX2*) were also up-regulated, while the transcripts to abscisic acid and brassinosteroids exhibited down-regulation in CA-H comparison (Supplementary Table S6).

In the MCPCA-H and MCPCA-CA comparisons, only a small number of transcripts to hormonal metabolism and transcription factors maintained the same behavior for the two growing seasons (Supplementary Table S6 and S7), exhibiting the environmental effect. Among the few transcripts that were expressed differentially, the ones encoding ethylene receptors were down-regulated in MCPCA-CA comparison (Supplementary Table S6). This difference in the behavior according to the year of harvest in the presence of 1-MCP was also observed in transcripts for lipid metabolism, as mentioned previously. Storch et al. (2017) through a correlation network analysis, demonstrated that a set of transcripts associated with oxidation and reduction, the biological processes with predicted functions in hormonal response, were influenced by the growing season, in apples treated with 1-MCP combined with CS.

In the case of TFs (Fig. 6; Supplementary Table S7), we could observe that certain ethylene-responsive factors (*ERF1* and *ERF/AP2*) were down-regulated in CA-H comparison, while *ERF 14*, *ERF 115*, *ERF 71*, and *ERF 72* were up-regulated. In apple fruit, two *ERF* transcripts, *MdERF1* and *MdERF2*, exhibited predominant and exclusive expression in ripe apple (Wang et al. 2007). Although the transcripts encoding for auxin homeostasis and response to auxin stimulus exhibited increased transcription after six months under CA storage, most of the sequences encoding auxin-responsive factors were down-regulated, except for *ARF5* and *ARF19* (Fig. 6; Supplementary Table S7). Certain transcripts to *MADS Box*, *Zinc Finger*, and *bHLH* family were up-regulated and down-regulated in CA-H comparison. On the other hand, *NAC*, *WRKY*, and *MYB* TFs were mostly up-regulated after CA storage, compared to fruit at harvest (Fig. 6; Supplementary Table S7). These results suggest a complex network mechanism for apple-ripening regulation after CA storage, with a balance of transcripts acting in positive and negative ripening responses. However, when 1-MCP was present in the comparisons (MCPCA-H and MCPCA-CA), most of the transcripts for TFs exhibited the same behavior as that of lipid and hormonal transcripts; that is, no coherent response was observed between the growing seasons, indicating environmental influences on the 1-MCP effect on these sets of genes.

The role of TFs mentioned above has been comprehensively elucidated in tomato (Shima et al. 2014). The authors observed that the initial ripening signal is caused by the upstream action of *RIN13*, where a *MADS-box* TF triggers the transition from the autoinhibitory production of ethylene (system 1) to autocatalytic phase (system 2) (Shima et al. 2014). The *MADS-box* has been related to the control of ripening events before the climacteric period and has been strongly associated with apple flesh firmness regulation (Ireland et al. 2013). In flesh fruit, it was also demonstrated that *MADS-box* gene suppression led to an increase in auxin concentration and maturation delay (Ireland; Seymour et al. 2013). In this investigation, certain *MADS-box* genes were also found to be down-regulated in CA compared to harvest; however, most of the genes were up-regulated at this condition (Fig. 6), suggesting the presence of active mechanisms to restore ripening in the apples submitted to a technology that inhibits ethylene biosynthesis.

It has also been suggested previously that auxin plays a role in fruit ripening and maturation in association with ethylene, although the molecular function and their interaction in fruit ripening are not well-elucidated (Busatto et al. 2017). In several climacteric fruits, auxin level has been hypothesized as an influencing factor for ethylene production, through the induction of ACC synthase activity during the fruit ripening (Busatto et al. 2017). However, a reduction in the auxin concentration before the beginning of fruit ripening appears to be necessary for climacteric fruit (Purgatto et al. 2002; Trainotti et al. 2007). Here, we could observe that the transcripts for maintenance of auxin homeostasis

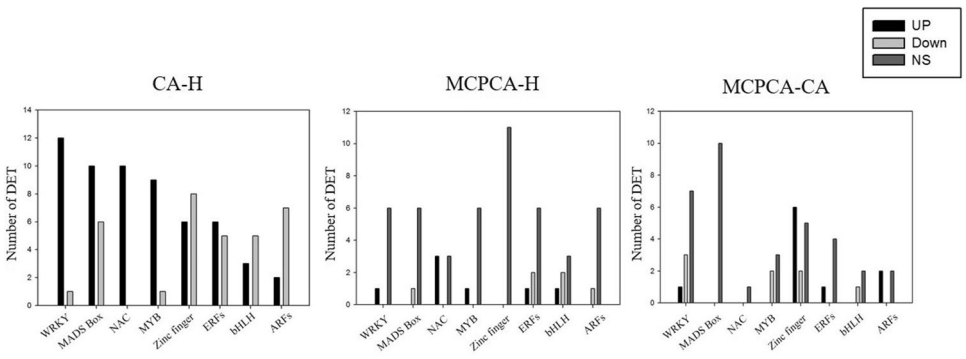


Fig. 6 The number of Differentially Expressed Transcripts (DET) of transcription factors. The comparisons correspond to CA storage versus harvest (CA-H), CA storage in the presence of 1-MCP versus harvest (MCPCA-H) and CA storage in the presence of 1-MCP versus CA storage (MCPCA-CA). Upregulated transcript (▬), downregulated transcript (▭) and non-significant expression (▬), based on $p < 0.01$ and BH < 0.05

and response to auxin stimulus (*SAURs*) (Supplementary Table S6), which have been reported to act in ACC synthase regulation (Trainotti et al. 2007; Tadiello et al. 2016; Busatto et al. 2017), were up-regulated after six months under CA storage.

In studies of Busatto et al. (2017), it was observed that the ethylene/auxin crosstalk appeared to be triggered by interference at the ethylene-receptor level. It has already been documented that post-harvest conservation technologies efficiently delay the general apple ripening, especially due to the hampered accumulation of ethylene (DeEll et al. 2016; Storch et al. 2017). However, despite the impairment of ethylene signaling, fruit attempts to restore the normal progression of physiological ripening. Authors have suggested that a parallel pathway involving *MADS Box* and *NAC* TFs operates when the ethylene perception is impaired, intending to stimulate late auxin synthesis to induce the ACC synthase and the final accumulation of ethylene (Busatto et al. 2017). Here also, we could observe a set of transcripts encoding ethylene receptors, *MADS Box*, *NAC* TFs, and auxin-responsive genes that were differentially expressed in apple after six months under CA storage. Therefore, we suggest that a parallel pathway might be operating to regulate ethylene signaling and biosynthesis.

Zhu et al. (2014) observed that 1-MCP treatment in tomato induced auxin-related genes (*Aux/IAA*) and higher levels of *NAC* TFs. Furthermore, the authors observed that repression of *NAC4* (KC454000) resulted in ripening inhibition, with decreased ethylene synthesis, reduced carotenoid accumulation, suppressed chlorophyll breakdown, and down-regulation of a variety of ethylene-associated and ripening-associated genes, suggesting that *NAC4* plays an essential role in tomato-ripening regulatory network (Zhu et al. 2014).

Based on an extensive research review and the present transcriptional data, we propose a model for ripening regulation after long-term CA storage, which begins with ethylene-receptor transcripts (Fig. 7). Here, we observed that several ethylene-receptor transcripts were up-regulated in CA-H comparison (Fig. 7). Genetic and biochemical studies have revealed that ethylene receptors act as negative regulators of ethylene responses, and that ethylene-binding inactivates them, leading to final ripening responses (Guo and Ecker 2004). Therefore, we hypothesized that the up-regulation of ethylene receptors after CA storage could result in a decrease in ethylene sensitivity, as the up-regulation of these receptors might lead to progressive desensitization of the system, and higher ethylene concentrations might be required to maintain the same level of response. The active state of ethylene receptors results in the repression of ripening induced by ethylene, and an alternative pathway appears to be operating to regulate fruit ripening, which involves *NAC*, *MADS Box* TFs, and auxin/ethylene crosstalk, regulating the transition of autoinhibitory ethylene synthesis (system 1) into autocatalytic phase (system 2) (Fig. 7).

In this study, we have identified numerous important TFs involved in apple ripening after six months under CA storage. Emerging research works suggest that relative functions of fruit hormones and TFs are not restricted to a particular stage, and a complex network is involved, controlling several aspects of fruit ripening. Most of these studies suggest crosstalk and signaling among the classic hormones and the recently identified classes of TFs and ripening regulators. The availability of expression arrays reveals the transcription of genes that are intimately associated with apple ripening. Nonetheless, additional research, to have a better understanding of the temporal and spatial expression of the genes that can be regulated by various pre-harvest and post-harvest treatments, are required.

The current works demonstrate a large-scale transcriptomic analysis of post-harvest apple ripening after six months under CA storage in the presence or absence of 1-MCP, revealing several candidate regulatory genes. Our results indicated alterations in several metabolic pathways, including cell wall biosynthesis and degradation, photosynthesis light reactions, lipid metabolism, and hormonal interplay with transcription factors. The transcriptomic study of apple under long-term CA storage has provided further insight into the ripening-regulating processes occurring under these conditions. The expression of a specific set of genes, such as ethylene receptors, *MADS Box*, *NAC* family, and *SAURs* (small auxin upregulated RNA), suggested the existence of crosstalk among ethylene, auxin and transcription factors regulating the apple ripening. Transcripts to cell wall biosynthesis and those related to membrane integrity, encoding *LHCs*, and thylakoid glycerolipids, were maintained by 1-MCP treatment combined with CA storage. Taken together, the results demonstrate a complex network influencing apple ripening submitted to post-harvest storage under CA after treatment with or without 1-MCP. Furthermore, this study also allowed for the identification of key apple quality genes that could guide further studies of genetic improvement of the apple tree.

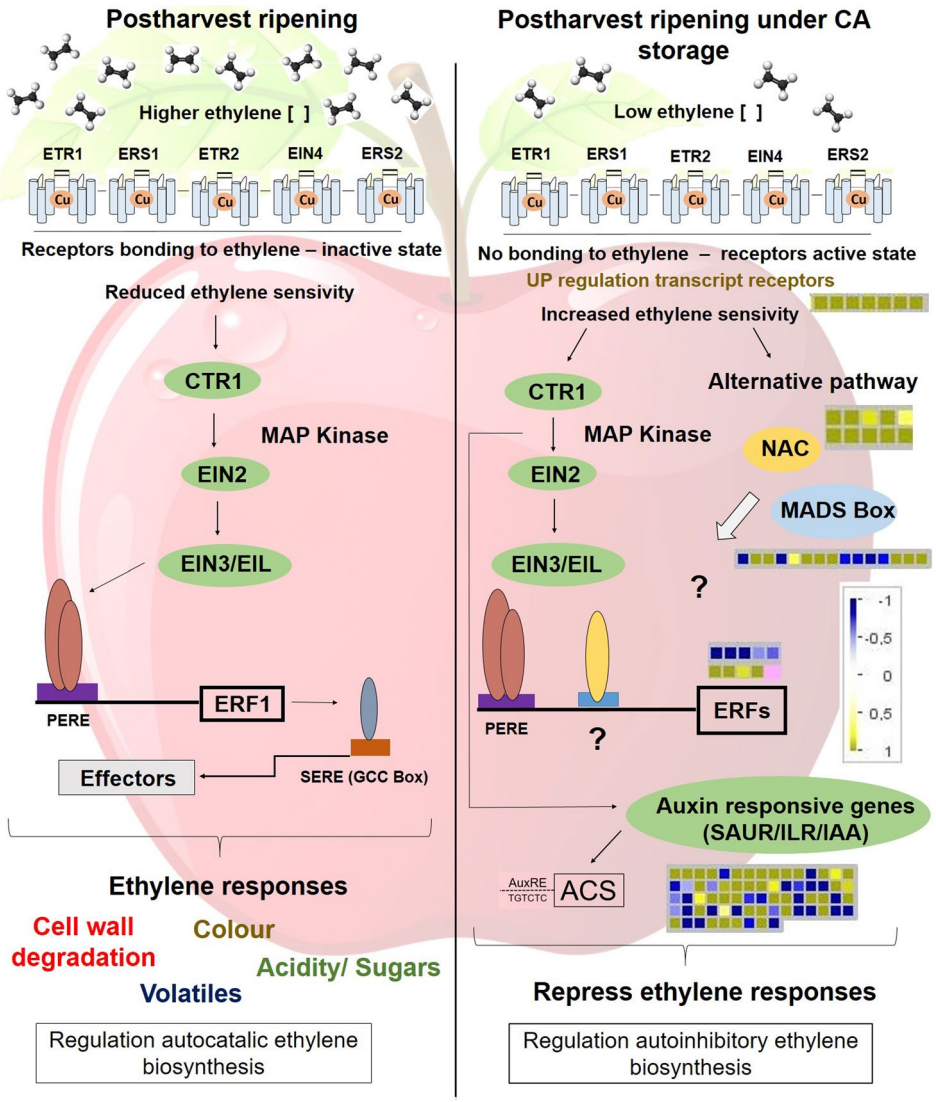


Fig. 7 Schematic representation of ethylene-signaling pathway model in postharvest fruit and regulation of transcript expression. **a** A family of ER-associated receptors (ETR) perceives ethylene gas. Ethylene binding is proposed to inhibit receptor function. The binding of ethylene to the receptors (ERSs and ETRs) turns off the active suppression exerted on CTR1, allowing the ethylene responses to occur. A MAPK module is proposed to act the downstream of CTR1. The downstream components in the ethylene pathway include several positive regulators (EIN2, EIN3, EIL, and transcription factors). The level of EIN3 protein is controlled by ethylene, possibly via the proteasome (Ub/26S). Several EREBP transcription factors are known to be immediate targets of EIN3/EIL, which can bind to a primary ethylene response element (PERE) in the promoters of EREBP genes. Other EREBPs may act in a similar manner to integrate the actions of ethylene with developmental signals and/or other hormone signals. **b** The hypothetical model illustrating the postharvest apple ripening regulation after CA storage. A basal ethylene concentration is present, which should not be enough to entire binding with receptors leading them to stay in an active state and regulate overexpression. CTR1 is proposed to be activated by the unoccupied receptors via physical interaction with them leading the entire ethylene perception system to be compromised. An alternative pathway to regulate fruit ripening seems to occur, involving *NAC*, *MADS Box,* and auxin, regulating transition to autoinhibitory ethylene synthesis (system 1) to autocatalytic phase (system 2). Yellow and blue boxes mean transcript behavior up and downregulated, respectively, in CA storage versus harvest (CA-H) comparison

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Author contributions CFPN and IRO participated in the design, performed physiological and gene expression analyses, performed statistical data analyses, and drafted the manuscript. TTS assisted in the physiological analyses, gene expression analyses and drafted the manuscript. MO-B, J-PR and FL participated in gene expression analyses. CVR and CLG designed the experiments and drafted the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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