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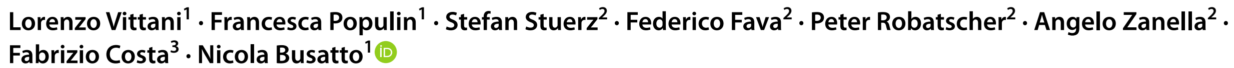
Candidate Gene Transcriptional Signature Unravels the Reprogramming Occurring in the Peel of Apple Fruit of 'Granny Smith' During Postharvest Storage



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Abstract

After harvest fruit are stored to preserve the quality features established during the on-tree development and maturation, ensuring thus a continuous availability of fresh fruit on the market. For certain fruit species like apple, storage can last for almost a year, especially when coupled with several strategies, such as the reduction of the oxygen concentration or the application of ethylene competitor molecules, like 1-methylcyclopropene (1-MCP). To guarantee the maintenance of the highest quality, the monitoring of the physiological processes ongoing during the postharvest ripening is compelling. For this purpose, 16 genes belonging to key fruit ripening pathways, such as the ethylene and the sugar/fermentation metabolism, have been chosen as potential markers for the molecular characterization of the major changes occurring in the fruit during storage. Among these genes, *ACS, PPO, PG1, RAP2-like*, and *ADH* exhibited the most significant differential expression across the various samples. Based on the transcriptional pattern, this set of genes constitutes a valuable molecular tool for a precise and reliable RNA-based monitoring of the postharvest ripening progression and fermentation process in apples. *PPO*, together with *S6PDH*, were furthermore employed to inspect the onset of the superficial scald in apple and resulted to correlate with the evaluation of the incidence of this disorder and the accumulation of the sugar alcohol sorbitol, known to play important protecting roles to chilling injuries. The assessment of the transcriptional signature of these elements can facilitate the development of gene expression markers suitable for a more informed investigation of the physiological progression of the postharvest ripening in apples, ultimately leading to the promotion of high-quality stored apples, extending storage time while minimizing postharvest disorders and fruit loss.

Keywords Apple · Postharvest · Transcriptomics · Superficial scald · Hypoxia · Markers · Ethylene

Introduction

To extend fruit quality and safety, temperature levels are lowered down during the postharvest storage. In apples, the prolonged storage at a temperature below 2 °C can also trigger the onset of a serious disorder known as superficial scald, which occurs in a cultivar-dependent fashion (Gong et al. 2021; Vittani et al. 2023; Watkins et al. 2000). This physiological phenomenon is associated with structural disorganization of mitochondria and chloroplasts (Busatto et al. 2018) and is characterized by the development of necrosis on the hypodermal tissue, without, however, affecting the inner pulp (Gong et al. 2021). These symptoms could emerge during cold storage and worsen during the subsequent re-establishment of room-temperature ripening, negatively affecting the marketability of susceptible apple cultivars (Karagiannis

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et al. 2018). Several pre-harvest factors, as well as post-harvest conditions including genetic background, ripening stage at harvest and storage procedure may influence the incidence of the symptoms and their severity, causing significant economic losses for growers and a reduction in the consumers' appreciation (Gapper et al. 2017; Sevillano et al. 2009).

Superficial scald symptoms arise as an oxidative reaction following chilling injury phenomenon during storage, leading to the accumulation of oxidative compounds in the apple skin (Rowan et al. 2001; Wang 2016; Marc et al. 2020). The aetiology and regulation of this disorder have recently interested the scientific community, since many studies addressing this topic have been recently published (summarized in Cainelli and Ruperti 2019; Lurie and Watkins 2012), although its understanding is not complete yet. Early targeted metabolic studies assumed that this process is mainly triggered by the oxidation of the volatile sesquiterpene α-farnesene synthesized through the mevalonate pathway, which is mainly accumulated in the epicuticular wax layer (Watkins et al. 1995). Its subsequent autoxidation may afterwards lead to the production of highly reactive conjugated trienols (CTs), converted by non-enzymatic reactions into the ketone 6-methyl-5-hepten-2one (MHO) (Dias et al. 2020; Lurie and Watkins 2012; Rowan et al. 2001). It has also been hypothesized that these CTs may affect the integrity of cell membranes by influencing lipid composition (Abdallah et al. 1997). In parallel to this process, oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POX) can interact with their respective substrates, leading to the development of the necrotic areas typical of scald (Dias et al. 2020; Gapper et al. 2017). Previous studies revealed the close connection between α-farnesene and ethylene production, since the expression of *AFS1*, the last gene encoding enzyme in the α-farnesene biosynthetic pathway, was positively regulated by this hormone and repressed by the exogenous application of the ethylene competitor 1-MCP (1-methylcyclopropene) (Apollo Arquiza et al. 2005; Rupasinghe et al. 1998). However, as pointed out by Gapper et al. (2017), levels of CTs rise before the onset of symptoms and decrease as scald develop. Other studies reviewed the α-farnesene theory, suggesting alternative hypothesis focussed on the changes of lipid and phenol composition. To this end it was in fact proposed that one of the major variables governing membrane fluidity and functionality is the degree of fatty acid unsaturation (Lurie et al. 2005). During the onset of superficial scald, a loss in the bi-layer membrane integrity in the chloroplast occurs (Bain and Mercer 1963), causing the release of polyphenol oxidase enzyme (PPO) into the cytosolic space enabling, therefore, the interaction with phenolic substrates (mainly chlorogenic acid) normally stored in the vacuole (Nicolas et al. 1994). According to Ju et al. (1996) and Piretti et al. (1996), the polymerization of simple phenols and flavonoids is the responsible event causing the browning process (Busatto et al. 2018), which is the most evident symptom of superficial scald. The activity of PPO in wounded tissues is also believed to maintain, in a feedback control, the increased accumulation of phenolic compounds, playing an antioxidant and scavenging role fundamental to reduce oxidative damage provoked by the enhanced production of ROS radicals (Busatto et al. 2014; Di Meo et al. 2013).

In specific apple cultivars, such as 'Granny Smith', the onset of superficial scald can be nowadays efficiently prevented by the application of two postharvest strategies regularly applied to control the kinetics of fruit ripening, through the direct interference with the ethylene biosynthesis and perception, such as the application of 1-MCP or the control of the storage atmosphere by lowering down the oxygen concentration. Although both strategies commonly down-regulate the ethylene-dependent expression of *PPO* (Adams and Brown 2007; Taranto et al. 2017; Vittani et al. 2023), they are also distinguished by distinct preventing mechanisms. 1-MCP can in fact stimulate the accumulation of cryoprotectant agent such as the alcohol sugar sorbitol and the progressive accumulation of unsaturated fatty acids in cell membranes, increasing therefore the membrane fluidity in response to cold stress (Busatto et al. 2018). The use of controlled atmosphere has also been proved to be an effective method for the prevention of superficial scald in apples and the improvement of shelf-life, making it an attractive alternative solution to chemical applications. Controlled atmosphere is to date generated through different strategies, among which the most innovative and sophisticated is represented by the dynamic controlled atmosphere (DCA) (Zanella 2003), in which the concentration of oxygen is dynamically reduced around 0.4 kPa by detecting chlorophyll fluorescence or by the continuous assessment of the respiration rate of the fruit (respiratory quotient RQ; Verreydt et al. 2022), resulting an effective strategy in the prevention of the cellular membrane breakdown (Cristiana et al. 2018; Kawhena and Fawole 2021; Mahajan et al. 2014; Riaño et al. 2022). The low level of oxygen delay the progression of the general ripening and prevents the onset of superficial scald through a direct control on the biosynthesis of ethylene, since ACC oxidase (ACO), the last ethylene forming enzyme, requires oxygen to catalyze the conversion of 1-aminocyclopropane-1-carboxylate (ACC) into ethylene in its final form. The application of low level of oxygen during storage (hypoxia) can also stimulate an acclimation mechanism triggering the activation of important genes known to play a role during anaerobiosis, such as *pyruvate decarboxylase* and *alcohol dehydrogenase*. Although this mechanism, genetically coordinated by the *ERF-VII* class transcription factor, was originally disclosed in submerged tissues of Arabidopsis (Giuntoli and Perata 2018; Licausi et al. 2011; Weits et al. 2014), a conserved role has been recently also unraveled in

low oxygen stored apples (Cukrov et al. 2016; Vittani et al. 2023). The transition from an aerobic to an anaerobic type of respiration, and the prolonged maintenance of fermentation, can however also negatively influence the quality of a fruit for the accumulation of acetaldehyde. This compound, acting as an electron receiving molecule, contributes to the regeneration of NAD^+, and its reduction through a hydrogenation process leads to the formation of ethanol, with the consequent production of derived off-flavour type of volatile organic compounds (VOCs).

To monitor this transition, we assessed the transcription profile of a core-set of candidate genes involved in ethylene, sugar metabolism, hypoxia, and superficial scald pathway, in skin tissue of 'Granny Smith' apples cold stored in both, normoxia and hypoxia conditions, with and without the application of 1-MCP. Furthermore, we considered their employment as potential novel molecular markers for an RNA-based routine monitoring assessment. By efficiently inspecting the physiological modification triggered by these postharvest strategies, the use of reliable functional markers can represent a valuable methodology to gain knowledge in the prevention of superficial scald and the control of the fermentative metabolism in apple during storage.

Material and Methods

Fruit Sampling, Storage Conditions and Evaluation of the Incidence of Superficial Scald

For the purpose of this investigation the cultivar 'Granny Smith' was chosen due to its well-known susceptibility to superficial scald (Suppl. Table 1), making it the reference cultivar for investigating the occurrence and regulation of this major postharvest disorder for pome fruits (Lurie and Watkins 2012). Apples were collected from full bearing adult trees at the experimental orchard of the Research Centre Laimburg in Northern Italy. The plants were grown following standard cultural and disease management strategies. The harvest time was determined after assessing fruit firmness and starch degradation. Specifically, apples were harvested at the of firmness of 6.8–7.7 kg/cm^2 and a starch iodine index value of 1.7–2.5 (based on a 1 to 5 scale). The harvested apples were subsequently divided into four batches, described as follow:

(i) Batch of apples stored under regular atmospheric conditions (RA) at 1.3 ℃ with 92% relative humidity for 6 months (CTR_RA).
(ii) Batch of apples treated with 1-MCP for 24 h, following AgroFresh instructions, and then stored under regular atmospheric conditions (1-MCP_RA) at 1.3 °C for 6 months.
(iii) Batch of apples stored under dynamic controlled atmosphere based on chlorophyll fluorescence (DCA-CF, henceforth CTR_DCA) with low oxygen concentration (0.2 kPa O_2 and 1.0 kPa CO_2) at 1.3 °C for 6 months.
(iv) Batch of apples treated with 1-MCP for 24 h, following AgroFresh instructions and stored under dynamic controlled atmosphere based on chlorophyll fluorescence (DCA-CF, henceforth 1-MCP_DCA) with low oxygen concentration (0.2 kPa O_2 and 1.0 kPa CO_2) at 1.3 °C for 6 months.

After six months of storage, a second set of four complementary batches of apples were also subjected to a post-storage shelf-life (SL) period, by keeping the fruit at room temperature (20 °C) for one week. Apples were further visually inspected for superficial scald symptoms, and samples of skin tissue were collected from pools of five apples representing each condition. These samples were immediately frozen in liquid nitrogen and stored at -80 °C for further analysis, including gene expression and metabolite profiling. Three replicates were prepared for both transcriptomic and metabolite analyses for each sample.

RNA Isolation and Candidate Gene RT-qPCR

The total RNA was extracted from the sampled fruit skin in each condition using the Spectrum™ Plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). RNA quantification and quality control were performed using the NanoDrop ND8000™ spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the Tapestation with the RNA ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA).

For cDNA synthesis, 1 µg of RNA from each sample was transcribed using the SuperScriptTM III™ cDNA Synthesis kit (Invitrogen). RT-qPCR was conducted on the ViiA7 PCR System™ (ThermoFisher Scientific) following the thermal profile described by Busatto et al. (2019b).

The transcription profiling considered 16 candidate genes (Suppl. Table 2) involved in key pathways related to fruit quality, anoxia response and superficial scald development in apple (Busatto et al. 2014; Vittani et al. 2023; Zubini et al. 2007). In particular, the list of genes related to anoxia and sugar pathways were selected according to the RNA-seq results published by Vittani et al. (2023), and representing a subset of reliable candidate genes for monitoring the response to low oxygen stress in apple. Actin gene (Md8283) was employed as housekeeping (Botton et al. 2011).

The relative gene expression was represented as the mean of normalized expression, considering three independent Ct values. The normalized value for each sample was calculated using the "Q software" (Muller et al. 2002; Simon 2003).

Analysis of Sorbitol

For the analytical determination of sorbitol, peel tissues from each sample included in this experimental design were grinded in liquid nitrogen and stored at − 80 °C until final usage. Before metabolite extraction, tissues were lyophilized under vacuum (0.05 mbar) at − 40 °C and gradually brought to 20 °C with the following steps: − 40 °C for 1 h, increased of 1 °C/min until − 25 °C and after 30 min the temperature was increased by 1.5 °C/min until 20 °C. The freeze-dried samples were homogenized, and 30 mg were weighted and spiked with 40.0 µL of Fucose solution (5,000 mg/L) used as internal standard, and 1 mL of 80% Ethanol was added for extraction. The samples were shaken for 1 h with a thermomixer at 750 rpm at room temperature and further centrifuged for 10 min at 10,000 rpm. Another aliquot of 1 mL of 80% Ethanol (VWR International; Radnor, PA, USA) was added for a second extraction as described before. The combined extraction solutions were diluted to a volume of 20.0 mL with MilliQ water and filtrated through a 0.2 µm PTFE-filter before analysis. The analysis of sorbitol was carried out adopting the procedure reported by Eisenstecken et al. (2015), employing an ion chromatograph (IC) with pulsed amperometric detection (HPAE-PAD) to quantify the concentration of sorbitol. The analysis was performed with an ICS-5000 (Thermo Scientific Dionex, Sunnyvale, CA, USA), while the separation was achieved by a Dionex CarboPac PA1 Analytical column (4 × 250 mm) and a Dionex CarboPac PA1 Guard column (4 × 50 mm). The calibration curve of sorbitol covered a range between 1 and 100 mg/L. An isocratic condition of 40 mM sodium hydroxide (Sigma–Aldrich; St. Louis, MO, USA) was used for 17 min for the chromatographic separation of the sugars, followed by a regeneration of the column with 200 mM sodium hydroxide for 10 min and 3 min of 40 mM, for a total of 30 min. The flow rate was set at 1.2 mL/min, while the injection volume was 20 µL with the column temperature set at 30 °C. An Au on PTFE disposable working electrode and a pH-Ag/AgCl reference electrode were used. Sorbitol in samples extracts was quantified using the external calibration curve. The results were normalized for the IS and the sample weight considering the water loss during lyophilization. Sorbitol in apple samples is expressed as mg g^{-1} fresh weight. Sorbitol standard were purchased from Sigma–Aldrich (St. Louis, MO, USA). If not stated differently, MilliQ water was used. Data elaboration was conducted with the Chromeleon Software package (version 7.2, Thermo Scientific Dionex, Waltham, MA, USA).

Data Analysis

Data and statistical analyses were analyzed using R.4.3.1 (R Core Team (2017), R Foundation for Statistical Computing, Vienna, Austria). For the computation of the PCA, FactoMineR, factoextra and ggplot2 packages were used, while the heatmap was elaborated using pheatmap package. The impact of storage conditions on both gene expression profiles and sorbitol accumulation was evaluated through a two-way analysis of variance (ANOVA) and Tukey's (HSD) test. All statistical tests were performed at a significance level of $\alpha = 0.05$.

Results

Candidate Gene Expression Analysis

The global expression of the array of 16 genes selected for the purpose of this work and representing ethylene synthesis, signalling and ethylene-dependent processes, in addition to respiration and fermentation metabolism, was initially illustrated through a hierarchical heatmap (Fig. 1a). The genes were divided into four different k-clusters based on their expression patterns. The first group consists of genes showing higher expression during the cold storage period in samples treated with 1-MCP or subjected to DCA conditions (*ADH*, *GPI*, *PFP*). The second cluster comprises genes that are induced by low oxygen conditions or 1-MCP treatment, both after six months of storage as well as during the post-storage shelf-life ripening (*ARF4*, *PDC*, *S6PDH*, *INV*, *RAP2-4*, *RAP2-13*). The third k-cluster includes genes (*ACO*, *PPO*) exhibiting their maximum expression in the untreated samples (CTR_RA) and with a lower magnitude also after shelf-life (CTR_RA_SL). The fourth group comprehended genes belonging mainly to ethylene and ethylene related pathways (*ERS*, *ERF*, *AFS1*, *ACS*, *PG1*) and showing a higher expression mainly in the subset of samples assessed during the period of shelf-life.

The overall gene expression was furthermore employed to plot the distribution of the several samples over a 2D-PCA plot, computed considering the first 2 dimensions accounting together for 86% of the transcriptomic variability (Dim1: 55.3%; Dim2: 30.7%; Fig. 1b). The two components clearly distinguished the samples into two main groups characterized by the different temperature applied during storage. The samples assessed immediately after cold storage (6 M), regardless the type of treatment (DCA or 1-MCP), were projected towards the II and III quadrants of the PCA (Fig. 1b). In this group, moreover, the RA sample was located in the Dim1 negative area, while the 1-MCP, DCA and DCA + 1-MCP samples were instead distributed in the Dim1 positive area. The other group of samples, assessed after the post-storage shelf-life at the temperature of 20 °C (6 M + SL), were instead oppositely mapped over the 2D-PCA plot, towards the I and IV quadrants (Fig. 1b). As observed for the previous group, also for

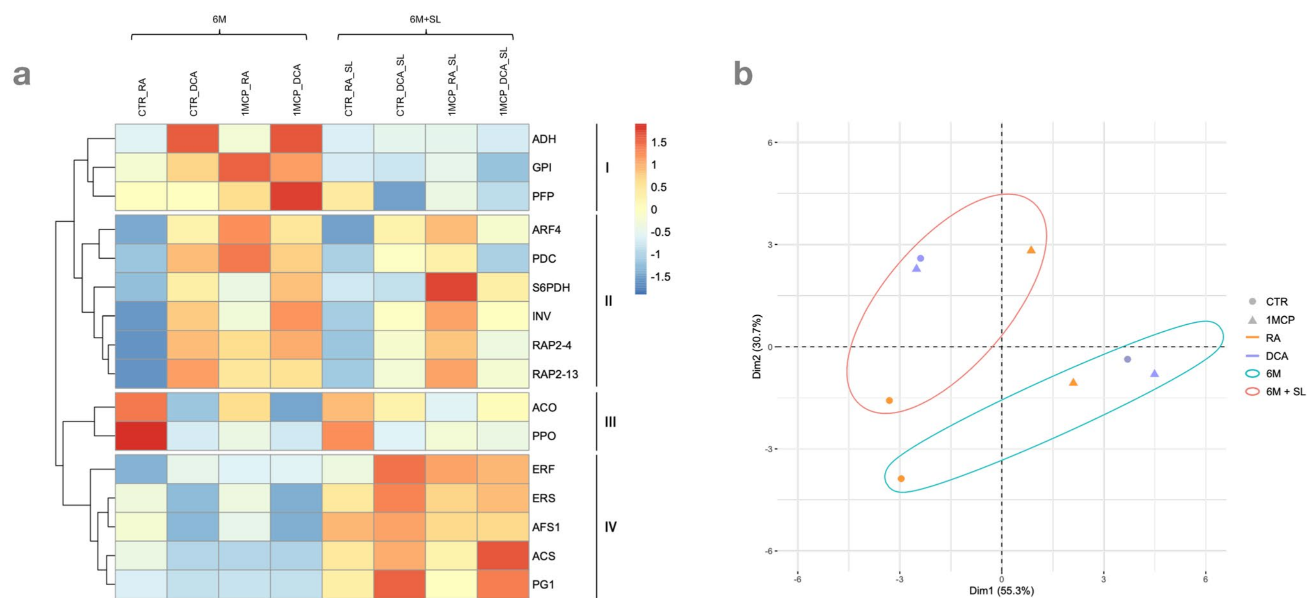

Fig. 1 **a** Heatmap illustrating the expression profile of the genes evaluated under the different storage conditions and shelf-life. The four clusters were defined on the base of the K-mean cluster analysis (Cluster I, II, III and IV). **b** 2D-PCA plot representing the overall variance across the experimental design using the expression profiles described in (**a**). The accompanying loading plot is comprehensively presented in Supplementary Fig. 1. *CTR* untreated fruit, *1-MCP* 1-MCP treated fruit, *RA* regular atmosphere, *DCA* dynamic controlled atmosphere, *SL* shelf-life. Colour scale and code are provided in the legends for both the panels (Color figure online)

the 6 M + SL set of samples a clear separation between RA and treated samples (1-MCP, DCA and DCA + 1-MCP) was observed but organized according to Dim2. In fact, while the 6 M + SL_CTR_RA sample was positioned in the Dim2 negative area, the group of the treated samples were in the Dim2 positive area of the plot. It is also interesting to note that the two samples considered as control (6M_CTR_RA and 6 M + SL_CTR_RA) were also closely located over the PCA plot and clearly separated from the rest of the treated samples. This different positioning was mainly assigned to the contribution of the loading projection of two main variables, represented by the expression of *ACO* and *PPO*, the two major elements involved in the biosynthesis of ethylene and the control of the superficial scald disorder (Suppl. Figure 1a and b). On the contrary, the distribution of the cold stored (6 M) treated samples (DCA, 1-MCP or their combination, DCA + 1-MCP) were instead characterized by a higher expression of the genes related to aerobic/anaerobic respiration (*PDC*, *ADH*), sugar metabolism (INV, GPI, PFP), acclimation to hypoxia (*RAP2-4* and *RAP2-13*) auxin (*ARF4*) and a key gene involved in the sorbitol metabolism (S6PDH) (Fig. 1a; Suppl. Figure 1B). The corresponding group of samples, assessed after the post-storage shelf-life (SL) ripening, was instead distinguished by a higher expression of elements related to ethylene (*ACS*, *ERS* and *ERF*) and ethylene related processes, such as cell wall dismantling (*PG1*) and biosynthesis of α-farnesene (*AFS1*) (Suppl. Figure 1b).

Expression of Ethylene Related Genes

The set of genes belonging to the ethylene domain were selected to represent different steps of the ethylene biosynthesis and signal transduction pathway, such as *ACS* and *ACO* (for the ethylene production), *ERS* and *ERF* (for the ethylene perception and signalling) and *PG1*, as an ethylene related gene. The expression of the two biosynthetic genes, *ACS* and *ACO* (Fig. 2a and b), although showing a distinct profile, resulted to be consistent with the approaches currently employed in the postharvest management to control the progression of the fruit ripening through the control of the hormone production. The two genes were in fact strongly repressed by both, the application of 1-MCP or the reduction of the oxygen concentration (hypoxia). The effect of the two strategies was also more evident during storage (6 M), than after the period of post-storage shelf-life. For *ACS* at 6 M + SL, the transcript accumulation was higher for the DCA and DCA + 1-MCP samples, with the latter sample showing the highest expression. The expression profile of *ACO*, consistent with *ERS* (Fig. 2c), showed that the lowering down of the oxygen concentration (DCA) during storage was more efficient than 1-MCP in turning down the mRNA level of both genes. The re-establishment of the room temperature stimulated the expression of this group of genes, in particular for *ACS* and *ERS*, where the expression in DCA and 1-MCP samples was even higher than in the 6 M + SL_CTR_RA sample. It is also worth noting that

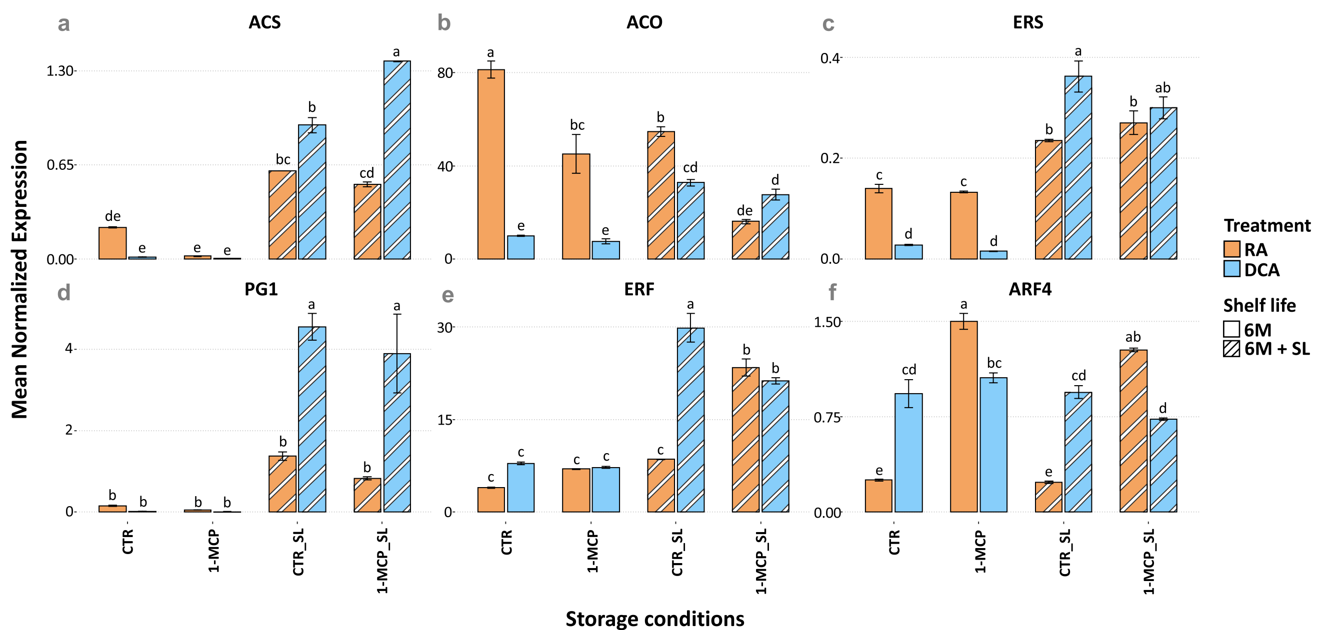

Fig. 2 Expression profile of **a** *1-aminocyclopropane-1-carboxylate synthase 1* (*ACS*), **b** *1-aminocyclopropane-1-carboxylateoxidase 1* (*ACO*), **c** *ethylene response sensor* (*ERS*), **d** *polygalacturonase 1* (*PG1*), **e** *ethylene response factor* (*ERF*) and **f** *auxin responsive factor 4* (*ARF4*). Error bars represent the standard error. Different letters above each column indicate significant differences (ANOVA coupled with the Tuckey HSD test was applied, $\alpha = 0.05$) (Color figure online)

PG1 (Fig. 2d), a major gene involved in the disassembly of the middle lamella and cell wall structure, and therefore in the control of the fruit softening process, was consistent with the profile of *ACS,* with a Pearson correlation value of 0.91. Similarly, to *ACS*, also *PG1* was severely down-regulated by the application of 1-MCP and the low concentration of oxygen, while its transcript level initiate to accumulate during the shelf-life period, reaching its maximum at the DCA samples in shelf-life. The higher expression of the ethylene related genes in 6 M + SL (including those stored at low oxygen or treated with 1-MCP) was also found for the *ERF* gene (Fig. 2e), known to be involved in the ethylene signal transduction. In fact, for this gene a linear and basal expression level for most of the samples was observed, while for the last three (DCA, RA + 1-MCP and DCA + 1-MCP in 6M_SL) a rapid increase was detected with a maximum expression detected for 6 M + SL_DCA. Recently, a cross-talk interaction between ethylene and auxin has been proposed to regulate the fruit ripening process also during the postharvest storage (Busatto et al. 2021). To this end, in this transcriptional survey, we also investigated the expression pattern of *ARF4* gene (Fig. 2f). This gene showed in fact an opposite pattern with regards to the ethylene biosynthetic genes, with a Pearson corelation of *r*: − 0.25 with *ACS* and *r*: − 0.61 with *ACO1*. *ARF4* was in fact more expressed in the samples treated to impair the ethylene accumulation (DCA, 1-MCP and 1-MCP + DCA) than the control (RA). For this gene, while the expression pattern in the two RA samples (at both 6 M and 6 M + SL stage) was comparable, the transcription profile of the treated samples at 6 M was slightly higher than at 6 M + SL.

To validate the actual role of accumulation of phenolic compounds and their oxidation operated by the polyphenol oxidase enzyme in the aetiology of the superficial scald disorder, in this investigation we assessed the transcript pattern of two principal genes involved in both pathways, such as *AFS1* (the major element involved in the biosynthesis of α-farnesene) and *PPO* (the gene encoding for the polyphenol oxidase enzyme) across the several samples included in the experimental design, since they showed a noticeable difference in the incidence of superficial scald. RA samples were in fact characterised by an important penetrance of the disorder's symptoms, while treatment with 1-MCP or storage at low oxygen concentration using DCA efficiently controlled its onset (Fig. 3). With regards to the development of the dark coloration on the apple skin, the expression of *AFS1* (Fig. 4A) was not consistent with the scald profile. In both storage time points (6 M and 6 M + SL) no specific regulation operated by 1-MCP was identified, while only at 6 M a slight effect was attributed to the reduced concentration of oxygen, which was however not confirmed in the 6 M + SL with the re-establishment of the regular room conditions. The expression of *AFS1* was in fact highly induced by the restore of room temperature (20 °C), with all the samples showing an enhanced gene expression similar to the pattern observed for *ERS*. On the contrary, the expression profile of

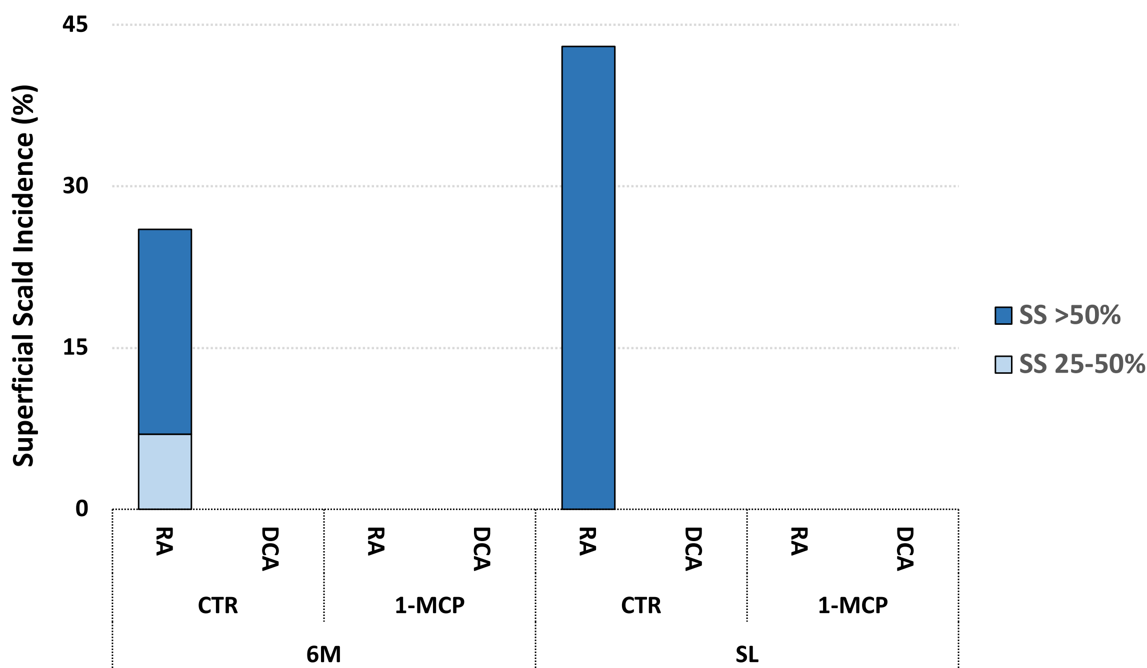

Fig. 3 Superficial scald incidence in 'Granny Smith' apples across the different storage conditions and after a post-storage period of seven days of shelf-life (SL) at room temperature. Fruit were stored following different strategies (*RA* room atmosphere, *DCA* dynamic controlled atmosphere and 1-MCP treatment). In light and dark blue are distinguished fruit with different superficial scald severity (SS 25–50%, and SS > 50%, respectively)

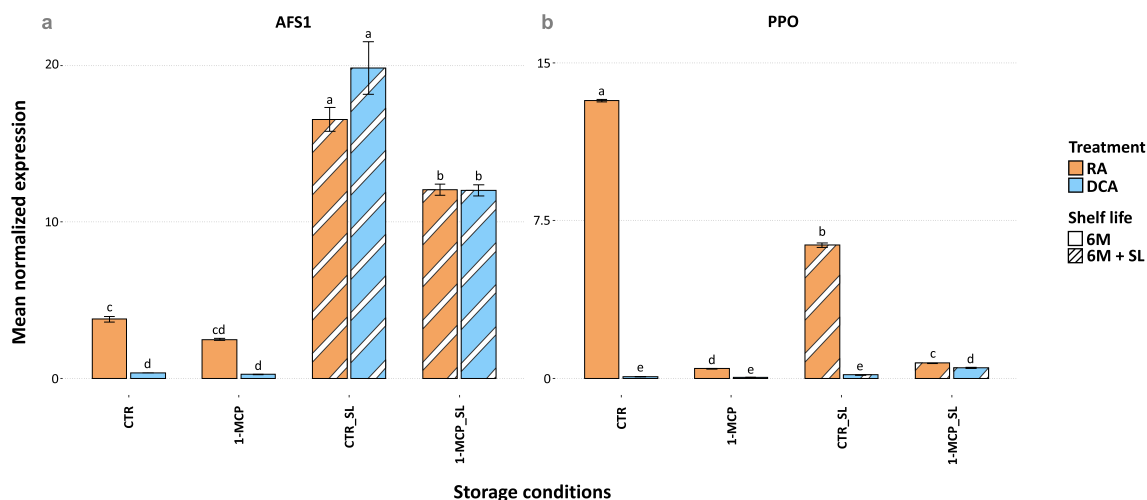

Fig. 4 Expression profile of **a** *alpha-farnesene synthase 1* (*AFS1*), and **b** *polyphenol oxidase* (*PPO*). Error bars represent the standard error. Different letters above each column indicate significant e differences (ANOVA coupled with the Tuckey HSD test was applied, $\alpha = 0.05$) (Color figure online)

PPO (Fig. 4b) showed a better consistency with the occurrence of the superficial scald symptoms. This gene was in fact highly expressed in the two RA samples (6 M and 6 M + SL) and severely down-regulated by the two strategies employed to control superficial scald (hypoxia and 1-MCP).

The samples included in this work were also employed to determine the concentration of sorbitol (Fig. 5a), due to its capability to mitigate chilling injuries, such as superficial scald and serving as a system to enhance cold resistance. At 6 M (with the continuous application of low temperature for six months) it was observed that the lowering down of oxygen and the application of 1-MCP contributed, with an additive effect, to increase the concentration of sorbitol with regards to RA samples, with a range of values from

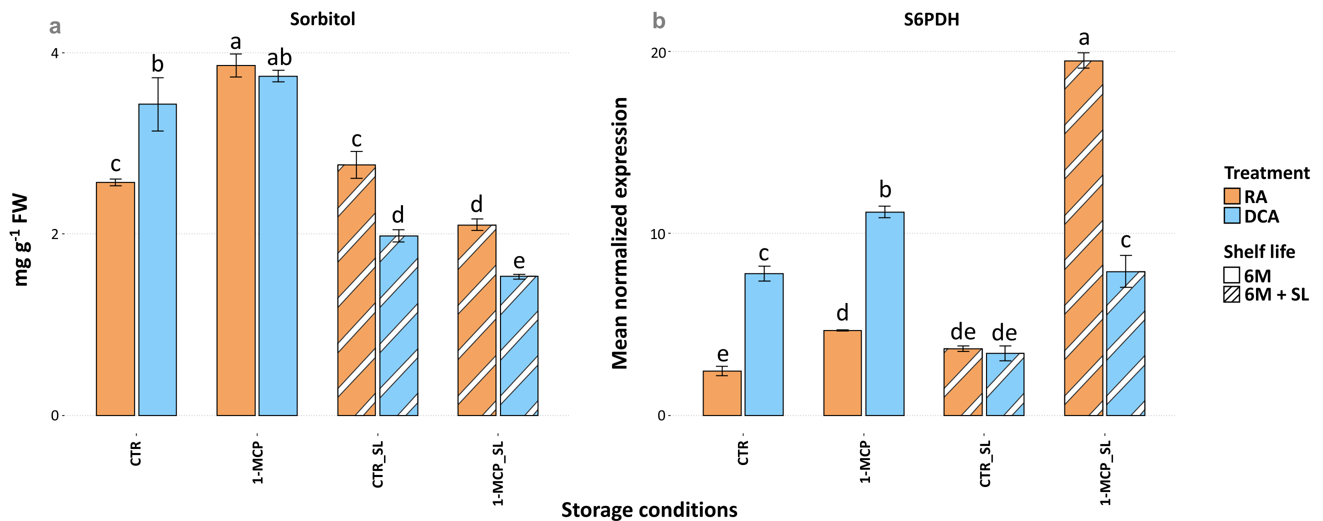

Fig. 5 **a** Accumulation of sorbitol in the skin tissue of the fruit stored under the different storage conditions and shelf-life. **b** Expression profile of *NADP-dependent sorbitol-6-phosphate dehydrogenase* (*S6PDH*). Error bars represent the standard error. Different letters above each column indicate significant differences (ANOVA coupled with the Tuckey HSD test was applied, $\alpha = 0.05$) (Color figure online)

1.53 mg g^{-1} (DCA_1-MCP_SL) to 3.86 mg g^{-1} (RA_1-MCP) and a fold change of DCA/RA = 1.34; RA_1-MCP/RA = 1.50; DCA_1-MCP/RA = 1.46. This effect was not detected at 6 M + SL, explained by the restoring to ambient temperature 20 °C for 7 days. The accumulation pattern of sorbitol, especially for the 6 M time point, was also consistent with the expression profile of *S6PDH*, a *sorbitol 6-phosphate dehydrogenase* playing a major role in the biosynthesis of this specific polyol. Consistent with the pattern of sorbitol, the expression of *S6PDH* was stimulated by both, DCA and 1-MCP, and with a more clear and consistent regulation at 6 M than at 6 M + SL (Fig. 5b).

With regards to the activation of the acclimation process to storage conditions by the fruit, we also examined, within the category of ethylene related genes, the expression of two *RAP* genes (*RAP2-4* and *RAP2-13*) involved in the adaptation process to hypoxia (Fig. 6a and b). Both genes were highly expressed in the 6M_DCA sample compared to the CTR, with a fold change of 4.08 and 4.63 for RAP2-4 and RAP2-13, respectively. It is also interesting to note that the

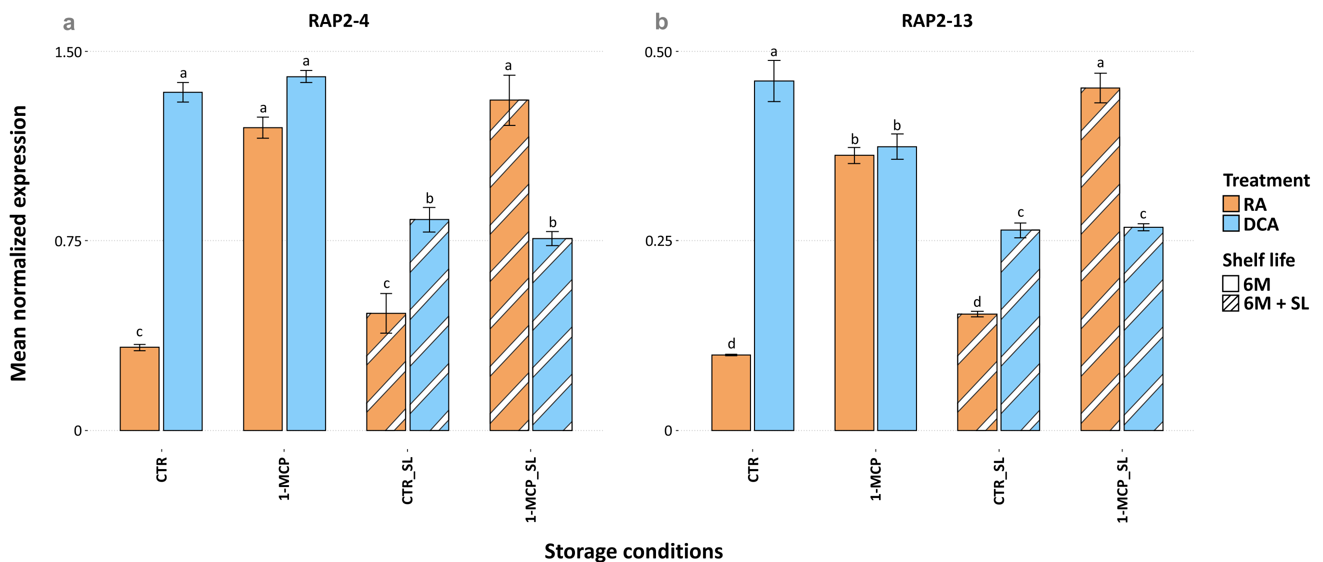

Fig. 6 Expression profile of **a** *related to apletala2-4* (*RAP2-4*), and **b** *related to apletala2-13* (*RAP2-13*). Error bars represent the standard error. Different letters above each column indicate significative differences (ANOVA coupled with the Tuckey HSD test was applied, $\alpha = 0.05$) (Color figure online)

treatment with 1-MCP also induced the expression of these elements, with a level comparable to DCA. Although with a modified expression profile, the same scenario was also observed in the 6 M + SL time point, but with a less evident regulation.

Expression of Genes Involved in the Metabolism of Carbohydrates and the Triggering of the Anaerobic Respiration

During storage apple fruit can undergo important modification of the sugar metabolism, in particular under hypoxia conditions, where the tricarboxylic acid pathway typical of the aerobic respiration shifts into the anaerobic fermentation process, potentially stimulated by the *RAP*-type of transcription factors.

To monitor the glycolytic route over the different storage conditions and treatments, the expression level of *INV* (*invertase*), *GPI* (*glucose-6P-isomerase*) and *PFP* (*PPi-dependent phosphofructotransferase*) were analysed (Fig. 7a–c, respectively). Both *INV* and *GPI* exhibited a higher expression level immediately after the cold storage, in the untreated sample under low oxygen condition (CTR_DCA) compared to untreated fruit stored in regular atmosphere (CTR_RA). Additionally, this effect was more pronounced for *INV,* which exhibited increased expression not only in the 1-MCP treated sample (1-MCP_DCA) but also during the shelf-life of control fruit (CTR_DCA_SL). The expression profile of *PFP*, however, showed a higher expression level in DCA compared to RA only in the 1-MCP treated sample (1-MCP_DCA) after 6 months of storage. The fermentation is a two-steps process, involving the conversion of pyruvate into acetaldehyde and further into ethanol, coordinated by the action of pyruvate decarboxylase and alcohol dehydrogenase, respectively. To investigate the reliability of potential molecular markers to characterize the fermentation process occurring in apple during storage, we assessed the transcription pattern of two genes encoding these two enzymes. The *PDC* (*pyruvate decarboxylase*) showed a higher expression in all the treated samples (DCA, 1-MCP, 1-MCP_DCA) compared to RA (Fig. 7d), with a higher magnitude at 6 M than at 6 M + SL. More consistent with the hypoxia condition was instead the expression of the *ADH* (*alcohol dehydrogenase*) gene. The transcript level of this gene was in fact specifically and highly induced in both samples stored under DCA conditions, regardless the treatment with 1-MCP, at the 6 M time points, with regards to the samples stored at regular atmosphere, RA and RA 1-MCP, with a fold change of 13.10 and 5.56, respectively (Fig. 7e). It is also worth noting that at 6 M + SL no significant differences were observed, since all the samples showed a uniform and a comparable basal gene expression level after 7 days of post-storage shelf-life ripening at ambient conditions.

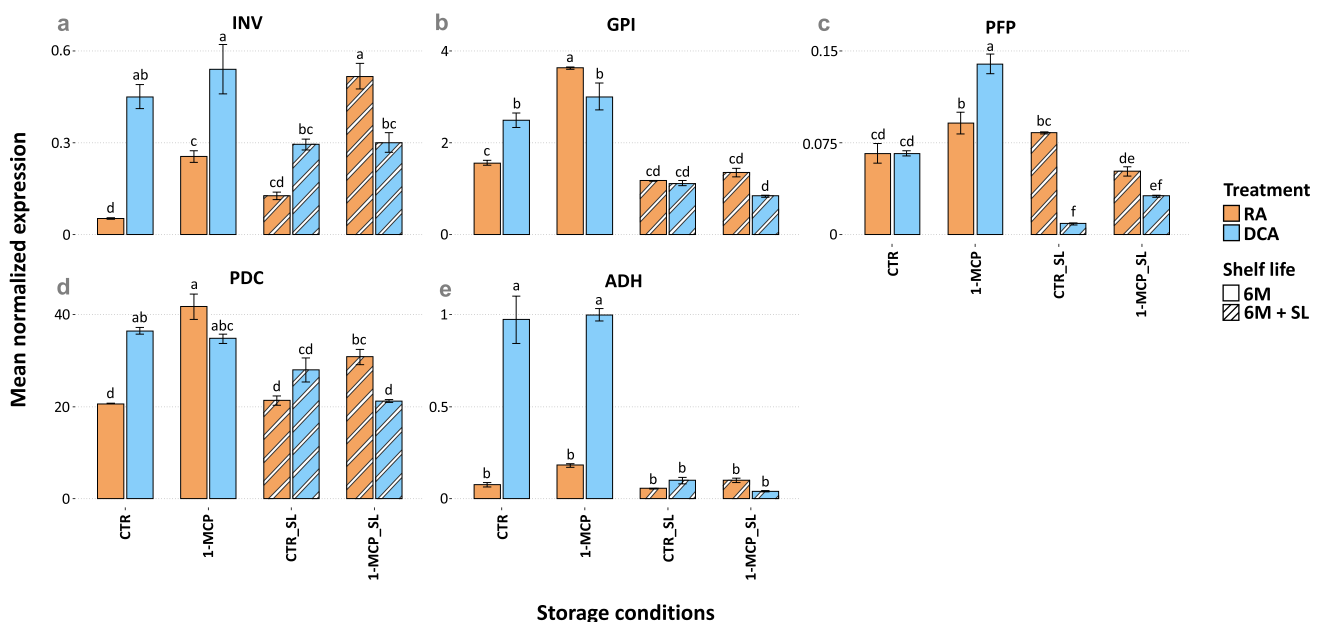

Fig. 7 Expression profile of **a** *invertase* (*INV*), **b** *glucose-6-phosphate isomerase* (*GPI*), **c** *PPi-dependent fructose 6-phosphate 1-phosphotransferase* (*PFP*), **d** *pyruvate decarboxylase 1* (*PCD*) and **e** *alcohol dehydrogenase* (*ADH*). Error bars represent the standard error. Different letters above each column indicate significant differences (ANOVA coupled with the Tuckey HSD test was applied, $\alpha = 0.05$) (Color figure online)

Discussion

The low temperature applied to promote fruit storability can also stimulate the occurrence of typical postharvest disorder, such as the superficial scald in apple. To prevent this phenomenon, in addition to low temperature, the postharvest technology can nowadays take advantage of molecules acting as competitor of ethylene (the principal hormone coordinating the progression of ripening in climacteric fruits; Busatto et al. 2019a; Lurie and Watkins 2012; Watkins 2006; Zhang et al. 2020), such as 1-MCP, or of the controlled atmosphere, which lower down the level of oxygen to the LOL point, the lower oxygen level tolerated by plant tissue (also known as anaerobic compensation point-ACP). Although the latter strategy received particular attention in the past years for its suitability in organic production, it can however induce fermentation process, which in turn can lead to the release of undesirable flavours derived by the accumulation of ethanol and related metabolites. The monitoring of the progression of the postharvest fruit ripening in apple for the promotion of high-quality standards, can be nowadays supported by the employment of reliable molecular markers suitable for an RNA-informed diagnostic tool. Several publications have already presented the application of genome-wide approach assessing the entire transcriptome in a single round of analysis, and specifically oriented to fruit quality and postharvest performance (Favre et al. 2022, 2023; Vittani et al. 2023). However, the generation and analysis of a large amount of data might result unfeasible or not affordable for a routine type of investigation. To this end, we have selected a core-set of 16 candidate genes potentially suitable to transcriptionally inspect the progression of the fruit ripening, the development of superficial scald and the monitoring of the fruit respiration metabolism in hypoxia condition. The general analysis of the transcriptional pattern of this set of genes through a multivariate statistical approach revealed the clear effect of the temperature in the control of ripening. To this end, it is worth noting that while the difference between RA_CTR and the treated samples during cold storage (6 M) was mainly attributed to the Dim1, after seven days at 20 °C (shelf-life) the same difference was instead attributed to the role of the Dim2, which explain about 30% of the total gene expression variability.

Monitoring of the Postharvest Fruit Ripening with Ethylene Related Markers

Among the set of candidate genes employed as marker in this survey, the first group was related to the ethylene pathway and ethylene related processes. In addition to elements known to be involved in the biosynthesis, perception and signalling of this hormone (*ACS*, *ACO*, *ERS* and *ERF*), we also considered other elements involved in the coordination of ethylene-dependent processes, such as the dismantling of the cell wall architecture (*PG1*), the crosstalk with other hormones, in particular auxin (*ARF4*), the regulation of superficial scald (*AFS1*, *PPO* and *S6PDH*) and the acclimation of hypoxia (*RAPs*). The two main genes related to the ethylene production (*ACS* and *ACO*) resulted to be controlled by both strategies employed to interfere with the hormonal production (1-MCP or DCA), which efficiently down-regulated the gene expression of these elements, but with differences. The expression of *ACS* was totally down-regulated by both approaches during storage (6 M), while for *ACO*, the DCA seemed to be more effective than 1-MCP in the control of the transcript accumulation. The application of 1-MCP reduced in fact the expression of *ACO* compared to RA sample (although still detectable) but did not show any relevant modification for the DCA sample. The dominant role of DCA (hypoxia) on the control of the expression of the *ACO* gene is consistent with the oxygen-dependent mechanism of action of 1-aminocyclopropane-1-carboxylic acid oxidase that require oxygen to convert the precursor ACC into ethylene. The role of these two postharvest strategies in controlling the gene expression was negligible in the post-storage shelf-life ripening for *ACS*. For *ACO*, instead, both 1-MCP and DCA resulted still efficient in the control of the transcript abundance, but with a less magnitude with regards to the 6 M stage. The return to room temperature and atmospheric condition can in fact restore a normal physiological condition, stimulating in turn the re-activation of the ethylene machinery towards the progression of ripening, especially in untreated fruit. Moreover, ethylene is known to interplay with other hormones (Van de Poel et al. 2015), and a recent work has underlined a possible interaction with auxin also during the postharvest phase (Busatto et al. 2021). To this end we have investigated the expression of *ARF4*, a transcription factor induced by auxin and taking part in this hormonal crosstalk (Busatto et al. 2021). The analysis of its expression pattern revealed an opposite trend with regards to ethylene related genes, being stimulated in both stages (6 M and 6 M + SL) by the two strategies employed to interfere with the ethylene production (1-MCP or DCA). The two contrasting patterns confirmed the hypothesis already formulated about a stimulation of the auxin machinery in a situation of ethylene impairment towards the re-establishment of a normal physiological progression of ripening. The low temperature applied during storage to control the general metabolism and needed to prevent over-ripening phenomena, can also induce harmful chilling injuries type of disorder, such

as superficial scald. The two strategies employed to control the general ripening (DCA and 1-MCP) are also known to efficiently control this disorder, especially in 'Granny Smith'. To better disclose the mechanism underneath its regulation, several works have been published (Busatto et al. 2014; Busatto et al. 2019a; Farneti et al. 2015; Vittani et al. 2023) and reviewed the role of the oxidation of chlorogenic acid by polyphenol oxidase as the main process leading to the onset of this disorder. Since the samples used in this experimental plan were distinguished by a different incidence of the disorder between asymptomatic and symptomatic tissues, we selected two representative genes to be used as markers for the superficial scald symptoms onset. The first element was *AFS1*, the main gene involved in the production of α-farnesene, a sesquiterpene known to play a role in the aetiology of superficial scald, while the second was *PPO*, the gene encoding for the polyphenol oxidase enzyme. By the inspection of the two expression patterns, the profile of *PPO* resulted to be better correlated to the actual development of superficial scald, being clearly over expressed in the two RA samples at both, 6 M and 6 M + SL, and strongly down-regulated by the two strategies adopted here to control the ripening and scald as well. *AFS1*, instead, was only slightly decreased by the application of DCA ad almost insensitive to 1-MCP, although its application efficiently prevented the occurrence of this disorder. The restore of room conditions (6 M + SL), moreover, stimulated the *AFS1* expression, but without showing any specific correlation with the level of symptoms observed among samples. The comparison of the expression data for the two genes proposed therefore *PPO* as a more reliable marker for the molecular monitoring of this disorder, validating the action of the polyphenol oxidase as the principal process governing this phenomenon. In addition to the control of the oxidation of the hydroxycinnamic acid by *PPO*, another proposed mechanism was the active increase of sorbitol accumulation to protect plant tissues from chilling injury events (Busatto et al. 2018). To validate this process, the concentration of sorbitol and the expression of *S6PDH* were also assessed on the samples defined here. Especially at the 6 M time point, both DCA and 1-MCP increased the accumulation of this sugar alcohol, in agreement with the expression of the *S6PDH* gene. This gene was also additionally regulated by the combination of the two preventing strategies, since the samples showing the highest expression of this gene and the highest accumulation of this sugar alcohol was the DCA sample treated with 1-MCP.

Definition of a Set of Markers for the Molecular Characterization of the Fermentation Process Induced by Low Oxygen Storage

Harvested fruit stored in controlled atmosphere with a minimal level of oxygen also initiated an acclimation mechanism to prevent the onset of specific disorders or to cope with low level of oxygen. The hypoxia condition, although necessary to extend storability through a modulation of the respiratory processes, can also promote the shift from the aerobic to the anaerobic respiration (fermentation), which can however influence the general fruit quality for the accumulation of ethanol and the subsequent generation of off-flavours. In order to monitor the transition towards the fermentation process, two transcriptional markers were initially assessed, *RAP2-4* and the *RAP2-13*. *RAP-type* of transcription factors have originally been identified in oxygen-deprived submerged tissues of Arabidopsis and barley as master regulators of processes addressed to initiate a hypoxia acclimation mechanism (Cho et al. 2021; Gibbs et al. 2015; Jethva et al. 2022; Luan et al. 2020). In aerobic conditions this class of transcription factor is normally associated to the plasma membrane to be afterwards translocated into the nucleus for triggering the expression of anaerobic respiration related genes (Giuntoli and Perata 2018; Licausi et al. 2011). From a genome-wide transcription assessment carried out in two apple cultivars ('Granny Smith' and 'Ladina'; Vittani et al. 2023) it was shown that *RAP2-type* of transcription factor are also activated in apple stored in controlled atmosphere distinguished by a low level of oxygen. Although hypoxia conditions are essential to slow down both the respiration and the production of ethylene, excessive reduction in the concentration of oxygen for an extended time can also stimulate the initiation of fermentative processes leading to a loss of quality. Both genes showed in fact a higher expression in all the treated samples, with an average fold change over the RA samples of 4.24 and 2.12 for the two time points (6 M and 6 M + SL), respectively. It is also interesting to note how the treatment with 1-MCP induced the expression of these genes also in samples stored at regular atmosphere (RA_1-MCP), hypothesizing a large spectrum of acclimation processes induced by the application of the ethylene competitor. In Busatto et al. (2021) and Vittani et al. (2023) it was already proposed how the application of 1-MCP could induce the expression of specific genes involved in particular metabolic pathways, such as those of the fatty acids. Consistent with the low oxygen-dependent mechanism, it is also worth noting how the response in 6 M + SL was about half with regards to 6 M, probably due to the re-oxygenation process occurring during the post-storage ripening at regular atmosphere. The oxygen-dependent mode of action demonstrated the

role of these genes as potential markers for inspecting the type of respiration ongoing in the fruit during storage. The end point of the reaction triggered by *RAP* is the activation of fermentative-related genes through a direct binding to hypoxia response promoter elements of genes promptly activated during the initiation of fermentative processes (Hess et al. 2011). Among these genes, key elements were represented by the *pyruvate decarboxylase* and the *alcohol dehydrogenase*, leading to the conversion of the pyruvate (generated from the glycolysis) to ethanol. Both genes were clearly expressed in all the samples stored in a deprivation of oxygen, but with the *alcohol dehydrogenase* showing the most evident and specific transcription pattern. The higher reliability of this gene was confirmed by the specific expression only in the DCA sample at 6 M, while at 6 M + SL, because of the effect of the re-oxygenation leading to the re-activation of the normal respiration, all the samples showed a similar and basal expression profile. The transcript analysis of these two genes underlined *ADH* as the more reliable and valuable gene to be used as marker for the RNA-based characterization of the initiation of anaerobic type of respiration in fruit during low oxygen storage.

During the fermentation process, the energy production efficiency is however low, resulting in only 2 ATP molecules generated per each molecule of glucose, in contrast to the 36 ATP molecules generated through glycolysis, the TCA cycle, and the mitochondrial electron transport chain in aerobic condition (Boeckx et al. 2019). Therefore, to support fruit survival in unfavourable conditions, the glycolytic flux is increased to provide a higher supply of glucose molecules for the fermentation process (Boeckx et al. 2019). The expression profiles of two key genes involved in the glycolysis pathway, *invertase* and *glucose-6p-isomerase*, confirmed this observation, exhibiting a higher expression level after six months of storage in the untreated sample under low oxygen condition (CTR_DCA), compared to untreated fruit stored in a regular atmosphere (CTR_RA). Typically, under anoxic conditions, pyrophosphate-dependent reactions are preferred to preserve ATP molecules whenever possible (Bailey-Serres et al. 2012). For this reason, we studied the expression level of a *PPi-dependent phosphofructotransferase*, a PPi-dependent enzyme, alternative to the ATP-dependent glycolytic enzyme *phosphofructokinase*.

Despite this premise, the expression pattern of *PFP* was not totally consistent with the low oxygen condition indicating how this gene is probably activated only in the early hypoxic response as previously observed by Cukrov et al. (2016), and the effect is no more visible after six months of cold storage, or at least in the untreated sample (CTR_DCA). While this alternative pathway may potentially mitigate ATP deficiencies during low oxygen stress, the precise extent of their impact remains uncertain and requires further investigation, as suggested by Mustroph et al. (2014).

Conclusion

The storage of apple is a crucial step of the entire production and commercialization process since it regulates the continuous marketability of fresh fruit on the market. Taken into account the most modern technologies and strategies nowadays applied to preserve the fruit quality during storage, apples can be maintained for almost a year-round period. The duration of the postharvest storage is nowadays longer than the pre-harvest life on the tree, and during this period the fruit is subjected to several physiological modifications, due to the changes of the environmental conditions employed in the storage room. The modification applied can, however, also negatively affect the quality of fruit, especially in susceptible cultivars. In order to avoid fruit loss and low quality, a continuous monitoring of the processes ongoing in the fruit during storage is essential to promptly modify the atmosphere composition for the prevention of fermentative processes and the onset of specific disorders. The definition of RNA-based markers can be a valuable tool for an easy and rapid inspection of the physiological state of a fruit. The core-set of genes identified and selected in this work might represent a valuable diagnostic system for the characterization of the physiological life of a fruit during storage. These genes were in fact selected in key steps controlling the postharvest ripening in storage, and their transcriptional signature can be used to determine the progression of the several postharvest ripening pathways leading to the final fruit quality. These markers can be thus considered as a valuable tool to improve the quality of stored apples.

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Declarations

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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