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Upregulation of polyphenol-related genes prevents 'skin burning' of well-colored 'Cameo' apples stored under stressful controlled atmosphere conditions

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ABSTRACT

'Skin burning' of 'Cameo' apples resulting in poorly colored fruit can occur in storage under high CO_2 condition. To elucidate possible reasons for this physiological disorder, we assessed the differential expression of polyphenol-related genes. Poorly colored and well-colored mature 'Cameo' apples were stored under either high (3%) or low (0.7%) CO_2 levels, both in combination with 1% O_2, and monitored for seven months for 'skin burning'. Samples were obtained by the end of storage period, and qPCR analyses were conducted using gene specific primers. We found expression levels of chalcone synthase (*MdCHS*), chalcone isomerase (*MdCHI*), anthocyanidin synthase (*MdANS*), flavonol synthase (*MdFLS*), dihydroflavonolreductase (*MdDFR*), and leucoanthocyanidinreductase (*MdLAR1*) genes to be substantially higher in well-colored compared to poorly colored apples. The delay in establishing the stressful controlled atmosphere (CA) storage condition (3% CO_2 level) led to significantly higher expression levels of *MdLAR1*, *MdCHI*, anthocyanidinreductase (*MdANR*) and flavanone 3-hydroxylase (*MdF3H*), which may explain the lower incidence of 'skin burning' by delayed CA fruit. On the other hand, after seven months in storage, the expression levels of phenylalanine ammonia-lyase (*MdPAL*), *MdCHS*, *MdCHI*, *MdDFR*, *MdFLS*, and *MdF3H*, were significantly higher in poorly colored injured apples, which reflect a feedback mechanism to synthesize more polyphenols to counteract the stressful storage condition.

1. Introduction

The market acceptance of red apples is largely determined by their peel color; better colored apples receive better acceptance (Saure, 1990). Moreover, the polyphenol concentration in peel is much higher than in flesh tissues, with colorless procyanidin as the predominant group, followed by quercetin glycosides (Tsao et al., 2003). However, the main anthocyanin of peels is cyanidin 3-galactoside (Ben-Yehuda et al., 2005). The biosynthesis of flavonoids starts with the phenylpropanoid metabolic pathway to produce 4-coumaroyl-CoA that combines with malonyl-CoA to give the backbone of all flavonoids. Further reactions encoded by a series of enzymes leads to the synthesis of flavanones, dihydroflavonols, anthocyanins, flavonols, flavan-3-ols and proanthocyanidins (Ververidis et al., 2007). At the molecular level, sets of genes are known to be involved in the biosynthesis of polyphenols, including genes encoding transcription factors or enzymes required for pigment biosynthesis (Goodrich et al., 1992; Honda et al., 2002; Kondo et al., 2002; Takos et al., 2006b). Some of the enzymes are phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone-3 b-hydroxylase (*F3H*), flavonol synthase (*FLS*), dihydroflavonol-4-reductase (*DFR*), leucoanthocyanidin reductase (*LAR*), leucoanthocyanidin dioxygenase (*LDOX*), anthocyanidin reductase (*ANR*), and UDP-glycose:flavonoid-3-O-glycosyltransferase (*UFGT*) (Takos et al., 2006b). However, the key enzymes controlling anthocyanin biosynthesis are believed to be those controlling the formation of cyanidin from dihydroquercetin (Ju et al., 1995). Considering that apple peel has much higher levels of cyanidin 3-galactoside relative to cyanidin 3-glucoside, it is possible to predict that the functional enzyme is UDPGal:flavonoid-3-o-glycosyltransferase (UFGalT) that transfers galactose to the 3-o position of flavonoids (Honda et al., 2002).

With respect to the effect of light on the polyphenol composition of fruit, Kim et al. (2003) reported that transcripts of *MdF3H*, *MdDFR*, *MdANS*, and *MdUFGT* were detected abundantly in the skins of cultivars with red skin, but rarely in non-red fruit, and that these

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genes were coordinately induced by light. Furthermore, shading of apples during cold conditions for two days led to a reduction of anthocyanins and an increase in photosensitivity of peel, leading to the hypothesis that anthocyanins may be adaptable light screens deployed to modulate light absorption in peels (Steyn et al., 2009). Telias et al. (2011) further found that striped areas of 'Honeycrisp' and 'Royal Gala' apples had high levels of both anthocyanins and transcripts of the major biosynthetic genes and the transcription factor *MYB10*. Rabinovich (2009) found also that the transcript levels of the biosynthetic genes and *MdMYB10* correlated well with the increase in the anthocyanin concentration in these stripes of 'Honeycrisp' and 'Royal Gala' apples. In the regulation of anthocyanin biosynthesis, ethylene interacts with light (Craker and Wetherbee, 1973). Moreover, it was found that exposure of dark-grown fruit to sunlight led to significant increase in *MdMYB1* transcript levels (Takos et al., 2006a). In this respect, Ban et al. (2007b) stated that the regulation of gene expression responsible for red coloration of apple peel is under the control of *MYB* transcription factors, which activate anthocyanin biosynthetic genes. In addition to that, Takos et al. (2006b) assessed the effect of sunlight on flavonoid accumulation and gene transcription of 'Cripps' Red' apples, and concluded that the transcription of most apple flavonoid genes is light responsive. Furthermore, Bakhshi and Arakawa (2007) found that light irradiation resulted in increased levels of phenolic acids, anthocyanin and flavonols, with no changes in the levels of flavanols, procyanidins and dihydrochalcones.

Consequently, the aim of this study was to explore the molecular basis of changes that may lead to the development of the physiological disorder 'skin-burning' in 'Cameo' apples, which appears usually upon storage under high CO_2 levels. In this regard, quantitative changes in the expression of genes that are involved in the biosynthesis of polyphenols from freshly harvested and stored 'Cameo' apples, either well-colored or poorly colored, were assessed.

2. Materials and methods

2.1. Plant material and storage condition

'Cameo' apples were picked with different blush color intensities (low intensity: <25% of surface with red coloration; high intensity: >50% of surface with red coloration). Fruit were picked from the orchard of the Competence Centre for Fruit Growing (KOB), Ravensburg, Germany. Thereafter, apples were stored at 3.0 °C under two CA conditions (1 kPa O_2 and <0.7 kPa CO_2, or 1 kPa O_2 and 3 kPa CO_2) using either a rapid or delayed establishment of CA conditions (delay of 21 days at 1.0 °C in regular air). After a seven months storage period, plus seven days shelf-life at 20.0 °C in air, samples were analyzed for CO_2 injury, which was assessed against a reference chart. Tissues from injured parts were shock-frozen using liquid nitrogen and kept at −80.0 °C for further analyses. For each treatment there were three replications, with eight apples for each replicate.

2.2. Colorimetric assay of polyphenolic antioxidants

Frozen samples were lyophilized. Subsequently, 50 mg lyophilized tissue from each replicate were dissolved in 3 mL of 80% methanol and further extracted for 30 min. Samples were centrifuged at 15,800 × *g* for 15 min at 4.0 °C, and the clear supernatants were kept protected from light at −20.0 °C. Upon analysis, 50 μL of the supernatant were diluted with deionized water (1:10), and 100 μL of Folin–Ciocalteu reagent (FCR) were added. The mixtures were mixed well with a vortex and kept for 3 min. Finally, 800 μL of sodium carbonate solution (7.5%) were added to each, and after vortexing, the mixtures were incubated for 60 min at room temperature. The absorption was measured at 720 nm using UV/VIS Spectrometer (PU8700, Philips). Pure ascorbic acid was used for calibration. Results are presented as mg ascorbic acid equivalent per g dry matter. Determinations were done on two pooled biological replicates per treatment, with two technical replicates for each biological replicate.

2.3. RNA-extraction and purification

Peels from 10 apples per replicate and sampling time were collected and directly frozen in liquid nitrogen. For analysis, peel tissues were ground to powder and RNA was extracted according to Chang et al. (1993) with some modifications. Eight grams of powdered tissue were added to 20 mL pre-warmed (65.0 °C) extraction buffer (100 mM Tris–HCl, pH 8.0; 25 mM EDTA; 2 M NaCl, 2% CTAB; 2% PVP 40; 2% β-*mercaptoethanol*) and incubated for 10 min at 65.0 °C. Thereafter, samples were centrifuged at 7740 × *g* for 10 min at room temperature. To 15 mL from the clear supernatant an equal volume of chloroform:isoamylalcohol (24:1) was added, vortexed, and centrifuged at 7740 × *g* for 20 min at room temperature. RNA was precipitated overnight at 4.0 °C by the addition of LiCl at a final concentration of 2.5 M. Following centrifugation (7740 × *g*) at 4.0 °C for 60 min, the pellet was washed twice with one volume of 70% ethanol, centrifuged for 10 min, and air-dried at room temperature. Finally, the pellet was suspended in RNase-free H_2O by heating at 65.0 °C for 5 min followed by vortexing. The insoluble particles were pelleted by centrifugation at 15,800 × *g* for 10 min at 4.0 °C. The quality and quantity of the extracted RNA were assessed by gel-electrophoresis and spectrophotometer, respectively. In addition to that, the RNA was purified further using the RNeasy® MinElute® Cleanup Kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA) and subsequently subjected to DNase treatment (RNase-Free DNase Set, Qiagen).

2.4. qPCR analysis

Taqman RT-PCR reagents (Applied Biosystems; Foster City, CA, USA) were used for the generation of cDNA for all samples. cDNA for each biological replicate was synthesized from 2 μg DNA-digested RNA using random hexamer primers. To assess the quantitative changes in the expression of the selected genes, gene-specific primers were designed (Table 1) based on sequences obtained from the NCBI database using Primer3 software (http://frodo.wi.mit.edu/primer3/), and those used in previous studies (Takos et al., 2006b; Espley et al., 2007). For each treatment there were two biological replicates, each with three technical replicates. Subsequent qRT-PCRs were carried out using the SensiMix kit from Bioline GmbH (Luckenwalde, Germany) on a LightCycler 480II from Roche Diagnostics Deutschland GmbH (Mannheim, Germany). The expression levels of the tested genes were calculated relative to transcript abundance of the reference gene (UBQ; U74358) employing relative quantification with efficiency correction (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Statistical analysis data are presented as means ± S.E.

3. Results and discussion

'Skin burning' of the apple fruit is a known physiological disorder with commercial relevance. Our results reveal that poorly colored fruit exhibited severe 'skin burning' injury, in particular after storage under high CO_2 levels (Fig. 1a). The assessment of total polyphenol capacity (Fig. 1b) did not differentiate clearly between

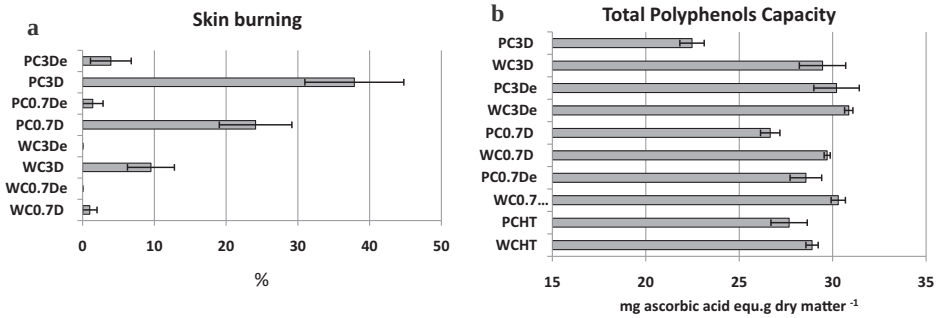

Fig. 1. Influence of fruit color and storage condition on (a) percentage of 'skin burning' and (b) total polyphenols capacity of 'Cameo' apples after seven months storage period. WC: Well colored (>50% blush); PC = poorly colored (<25% blush); HT = harvest time; 0.7 = storage at 0.7% CO_2; 3 = storage at 3% CO_2; D = direct establishment of CA-storage conditions; De = Delayed establishment of CA-storage conditions.

treatments, despite the clear reduction in total polyphenols in poorly colored fruit that were stored under the stressful conditions (3% CO_2). However, results of one of our concurrent studies (Harb et al., data not shown) clearly show that well-colored and healthy apples had significantly higher levels of cyanidin-3-galactoside, cyanidin-3-arabinoside, rutin, kaempferol glucuronide, hyperin, avicularin, and quercitrin, whereas poorly colored and/or injured peels had significantly higher amounts of p-hydroxybenzoic acid-glucose ester, caffeic acid glucose ester, phloridzin, chlorogenic acid, p-coumaryl-glucoside, (epi)catchin (epi)catechin isomers, phloretin-2′-xyloglucoside, and epicatechin. Taking into account that quercetin and cyanidin derivatives account for more than 80% of all antioxidants in apple peel, it is obvious that accumulation of these polyphenols in the peels of well-colored fruit may contribute highly to the prevention of 'skin burning'. Accordingly, investigating the connection between expressions of genes related to the biosynthesis of polyphenols and the incidence of this disorder is needed. In this sense, Fischer et al. (2006) reported that changes in polyphenol levels are usually accompanied by a distinct induction of gene expression by feedback regulation.

The expression profiles of the genes under investigation in this study are presented in Figs. 2–4. Since the low CO_2 (0.7%) treatments caused minimal damage, their expression profiles were omitted here. The first group of genes (Fig. 2) and *MdMYB10* (Fig. 4c) are highly expressed in well-colored fruit at harvest time compared to poorly colored fruit. This differential gene expression reveals that light is a major environmental factor controlling their expression. In this respect, Ban et al. (2007a) isolated 11 cDNAs from UV-B-irradiated apple skin, and three of these cDNAs were *MdCHS*, *MdF3H*, and *MdFLS*, which are putative flavonoid biosynthetic genes. The second group (Fig. 3) includes genes that showed high expression levels at harvest time, but differ in their expression profiles during the storage period. From this group, the *MdCHI*, *MdDFR*, and *MdF3H* genes were expressed highly in poorly colored and injured fruit after seven months storage. In the third group of genes (Fig. 4) the expression levels of *MdPAL*, *MdUDPG4E*, and *MdACS1* did not differ significantly between freshly harvested well-colored and poorly colored apples.

Table 1
Gene specific primers used to assess the expression of polyphenols-related genes of 'Cameo' apples.

MdPAL	DY255768.1	F: TGCACTGTGCCAAGCTATTGA R: TCAACAACCCTGAGGAGGTCT
MdCHS	X68977	F: GCAAGTGTTGTCAGATTACGG R: TGATACTGGTGTCTTCAAGCAG
MdCHI	X68978	F: GCTACAAATGCGGTGATAGAA R: TACCTTGGTTTCCAATTTTTCATT
MdANS	AF117269	F: GAAGATCATCCTTAAGCCACTG R: ATAATTTAGCACAACCCGCTTC
MdFLS	AF119095	F: CTTCTTACAGGGAAGCTAATGAA R: GAGGACATGGTGGGTAGTAGT
MdLAR1	AY830131	F: TAGAGCTACTGCAAGAGGAGG R: CCTCGAAGAAACCCTAGAAAC
MdANR	AY830130	F: GGCATCGAAGAAATATATGACCA R: AATTTACGGTAAGCCAGACAATA
MdDFR	AF117268	F: CAAGTACAGCTTGGAGGACAT R: TCCAAGCTGGTAAATGTAAAACA
MdF3H	AF117270	F: GAAGATGAGCAAGGATCTTGAG R: TTCCACAAAGAGCTTTCAAGTG
MdUDPG4E	GO521640	F: CCGTACCAAGCTTTTCCTTGA R: TAAGCTCAGGCAATCTTCCAA
MdACS1	DQ137849.1	F: TACTGAAACCGCTCTGGAAGAA R: CGTTTCTGGTCATTGTGGTGC
MdMYB10	DQ886416.1	F: TGCCTGGACTCGAGAGGAAGACA R: CCTGTTTCCCAAAAGCCTGTGAA
MdUBQ (reference gene)	U74358	F: CTCCGTGGTGGTTTTTAAGTG R: AGGAGGCAGAAACAGTACCAT

It is important to notice that *MdCHS* encodes the CHS enzyme, which in turn catalyzes the first committed step of flavonoid biosynthesis, namely the condensation of p-coumaroyl-CoA with malonyl-CoA molecules to produce chalcone. And that chalcone is considered as the precursor for a diverse set of flavonoids (Koduri et al., 2010). The expression profile of *MdCHS* is shown in Fig. 2a, which clearly indicates that freshly harvested and well-colored apples accumulated higher levels of *MdCHS* transcripts compared to freshly harvested poorly colored fruit. This is another indication for the crucial role of light in the biosynthesis of polyphenols. Ubi et al. (2006) reported that expression of *CHS* in bagged fruit was substantially depressed in skins, and that UV-B irradiation enhanced its expression. During the storage period the expression of *MdCHS* decreased dramatically in all treatments, despite significant differences between treatments. The highest expression level was found in poorly colored and injured apples. This can be seen as a defense mechanism to synthesize more precursors for the highly needed polyphenols to deal with the 'skin burning' injury. The significant difference between well-colored and poorly colored fruit at harvest time may be related to ethylene. El-Kereamy et al. (2003) reported elevated *CHS* transcript levels upon treatment of mature grape berries with ethylene. Also Ardi et al. (1998) found with avocado fruit that ethylene increased the activity of CHS.

The expression profile of *MdANS* (Fig. 2b) is relatively similar to *MdCHS*. Also here the well-colored fruit had more transcripts than poorly colored fruit. The significance of this gene is based on the action of the encoded enzyme ANS, which catalyzes the synthesis of anthocyanidins. Anthocyanidins are converted either by flavonol glycosyltransferase to the corresponding red 3-glycosylated anthocyanin product, or reduced through the action of ANR enzyme to the colorless epicatechin (Routaboul et al., 2012). In addition ANS may function as cyaniding-, pelargonidin-, or delphinidin-synthase (Shi and Xie, 2010). The significance of ANS is further enforced by the findings of Szankowski et al. (2009). In their study with apples, an almost complete blocking of anthocyanin biosynthesis

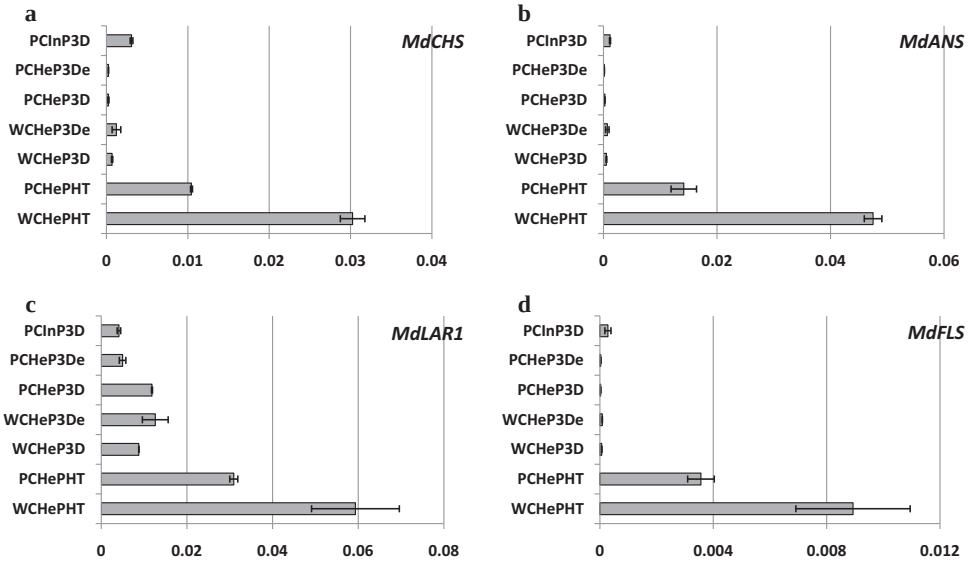

Fig. 2. The relative expression level of (a) chalcone synthase (*MdCHS*), (b) anthocyanidin synthase (*MdANS*), (c) leucoanthocyanidinreductase (*MdLAR1*), and (d) flavonol synthase (*MdFLS*) genes in well-colored (WC; >50% blush) and poorly colored (PC; <25% blush) 'Cameo' apples at harvest time (HT), and after storage for seven months under high (3%) CO_2 level. HeP = Healthy peels; InP = injured peels; 3 = 3.0% CO_2; D = direct establishment of CA-storage conditions; De = Delayed establishment of CA-storage conditions.

was achieved through the silencing of the ANS gene, which was accompanied by severe necrotic leaf lesions. It is likely that formation of leaf lesions, which is accompanied by very low levels of cyanidin 3-galactoside, is equivalent to the development of 'skin burning' by poorly colored apples reported in our study.

The expression profile of *MdLAR1* (Fig. 2c) shows the same trend as the other genes. Park et al. (2006) reported that *LAR* is up-regulated early in the ripening stage, which indicates that ethylene may induce its expression. After long-term storage, our results show clearly that healthy peel, irrespective of the coloration at harvest time, had significantly higher levels of *MdLAR1* transcripts than injured peel.

Another important enzyme is flavonol synthase (FLS), which catalyzes the biosynthesis of quercetin from dihydroquercetin; quercetin in turn can be glycosylated by glycosyl transferases (Newcomb et al., 2006). As shown in Fig. 2d, the transcript level of *MdFLS* in well-colored apples was much higher than in poorly colored ones. Literature about the influence of light and/or ethylene on the action of *MdFLS* is not available, but it is logical to assume that light influences its expression as well. Our results do not provide evidence for the influence of ethylene here, but Pelletier et al. (1997) found that *FLS* is an 'early' flavonoid gene in seedlings of *Arabidopsis*. In this sense, the expression of *MdFLS* may be independent of ethylene.

Fig. 3a shows the expression profile of *MdCHI* that is similar to previous genes at harvest time. However, poorly colored fruit, which suffered from 'skin burning' after storage under 3% CO_2 for seven months, accumulated much higher transcript levels of

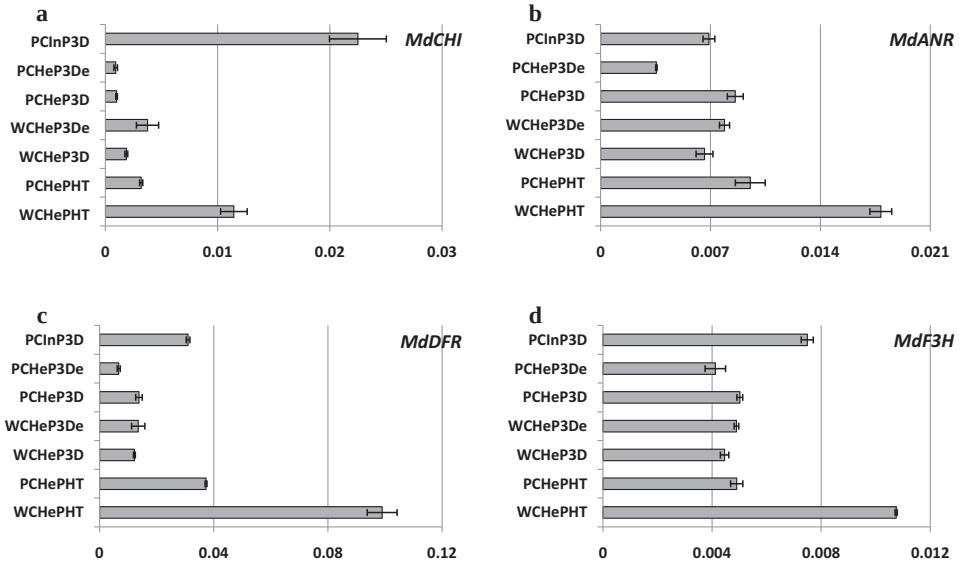

Fig. 3. The relative expression level of (a) chalcone isomerase (*MdCHI*), (b) anthocyanidinreductase (*MdANR*), (c) dihydroflavonolreductase (*MdDFR*), and (d) flavanone 3-hydroxylase (*MdF3H*) genes in well-colored (WC; >50% blush) and poorly colored (PC; <25% blush) 'Cameo' apples at harvest time (HT), and after storage for seven months under high (3%;) CO_2 level. HeP = Healthy peels; InP = injured peels; 3 = 3.0% CO_2; D = direct establishment of CA-storage conditions; De = Delayed establishment of CA-storage conditions.

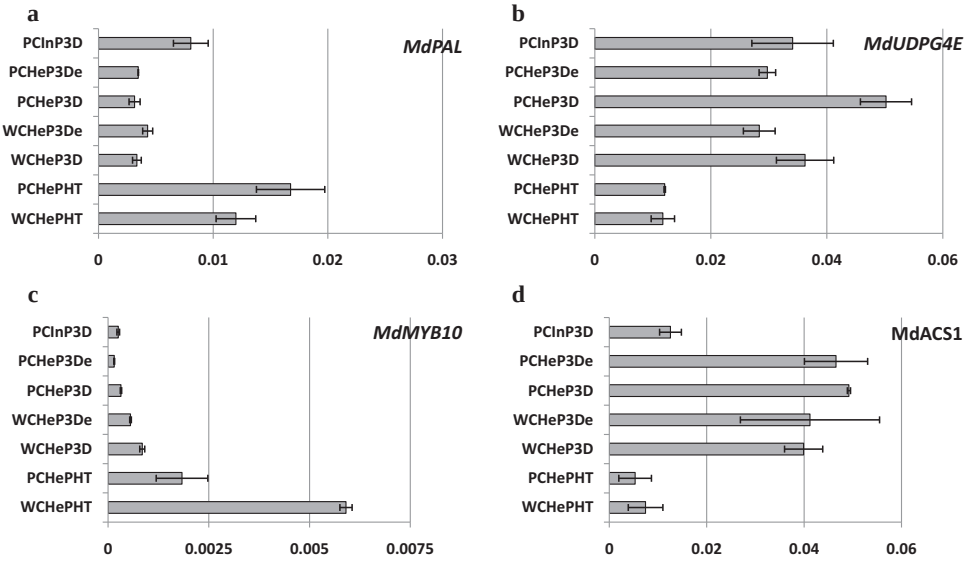

Fig. 4. The relative expression level of (a) phenylalanine ammonia-lyase (*MdPAL*), (b) UDP-galactose-4-epimerase (*MdUDPG4E*), (c) MYB transcription factor (*MdMYB10*), and (d) 1-aminocyclopropane-1-carboxylate synthase (*MdACS1*) genes in well-colored (WC; >50% blush) and poorly colored (PC; <25% blush) 'Cameo' apples at harvest time (HT), and after storage for seven months under high (3%;) CO_2 level. HeP = Healthy peels; InP = injured peels; 3 = 3.0% CO_2; D = direct establishment of CA-storage conditions; De = Delayed establishment of CA-storage conditions.

MdCHI than apples from all other treatments. The influence of light on the expression of *CHI* was assessed by van Tunen et al. (1988), who reported that *CHI* and *CHS* genes of *Petunia hybrida* are expressed in a light-regulated manner. With apples, Takos et al. (2006b) reported that fruit covered with bags failed to accumulate anthocyanins, whereas the expression of *MdCHI* increased by 240 fold in uncovered fruit. With regard to other genes in this group, it is obvious that the expression of *MdANR* (Fig. 3b), *MdDFR* (Fig. 3c), and *MdF3H* (Fig. 3d) shows similar patterns to *MdCHI*. In this respect, Vimolmangkang (2011) reported that overexpression of *ANR* resulted in the down-regulation of other genes that are involved in the flavonoid biosynthesis (e.g. *LAR* genes).

The last trend is related to the expression profiles of both *MdUDPG4E* (Fig. 4b) and *MdACS1* (Fig. 4d). Transcript levels of both genes were almost equal in both freshly harvested well-colored and poorly colored apples, which may indicate that these genes are not light-regulated. However, long-term storage led to differential responses, in particular with *MdUDPG4E*. Direct establishment of CA storage led to higher expression levels of this gene compared to the delayed CA conditions. Taking into account that UDP-galactose is the sugar component of cyanidin 3-galactoside, which accounts for around 80% of the total cyanidin glycosides in red apple peel (Lancaster, 1992), it might be possible to predict that a delayed expression of *MdUDPG4E* is preprogrammed to coincide with anthocyanin accumulation. Such synchronization was reported by Ban et al. (2009), and this synchronization may be due to ethylene action. In this respect, Ban et al. (2007b) reported that *MdUGE1* is highly expressed in mature fruit.

The expression of *MdMYB10* (Fig. 4c) correlated well with *MdCHS*, *MdANS*, *MdLAR1*, and *MdFLS*. Telias et al. (2011) reported that the transcript levels of the major biosynthetic genes for anthocyanins and *MYB10* also correlated well with anthocyanin concentration in stripes of 'Honeycrisp' and 'Royal Gala' apples. Further, Takos et al. (2006a) concluded that *MdMYB1* coordinately regulates genes in the anthocyanin pathway in apple skin.

The results clearly indicate that genes involved in the biosynthesis of various polyphenols respond differentially to light and/or high CO_2. The influence of high CO_2 on the quality of stored fruit is well studied. Argenta et al. (2000) found that storage of 'Fuji' apples under high CO_2 (3 kPa) and low O_2 (1.5 kPa) induced CO_2 injury, known as brown-heart. The down-regulation of some polyphenol related genes under high CO_2 level is reported in various studies. Shin et al. (2008) found that air-stored strawberries accumulated higher anthocyanins and flavonoids levels than fruit stored under high CO_2 levels. Furthermore, Fernández-Trujillo et al. (2001) reported that CO_2 treatment led to an increase in the succinate concentration, a compound that is usually associated with CO_2 injury. Moreover, Bodelón et al. (2010) reported that exposure of strawberry fruit to high CO_2 prevented the increase in total phenolics.

Another important aspect is related to the ripening hormone ethylene and its influence on the biosynthesis and accumulation of polyphenols. In this study, the levels of *MdACS1* (Fig. 4d) did not differ significantly between well-colored and poorly colored fruit. Since 'Cameo' apples already get their red color before the climacteric rise of ethylene, it is obvious that ethylene plays a minor role in polyphenol biosynthesis. Despite that, Schaffer et al. (2007) reported that genes involved in the early steps of phenylpropanoid pathway are ethylene-responsive. More interesting are the findings of El-Kereamy et al. (2003) with grape, a non-climacteric fruit, who found that ethylene triggers expression of genes involved in anthocyanin and phenolics syntheses.

The delay in establishing CA storage that resulted in lower incidence of 'skin burning' (Fig. 1a) can be correlated directly to the significant increases, upon delay, in the expression of *MdPAL*, *MdCHI*, *MdLAR1*, *MdANR*, and *MdF3H* in well-colored apples. In this respect, Argenta et al. (2000) and Colgan et al. (1999) reported that the delay in establishing CA storage led to a substantial reduction in CO_2 injury; they noted further that the susceptibility of apples to CO_2 injury is the highest during the first weeks of storage. However, the exact mechanism by which such delay reduces CO_2 injury of stored apples is unclear. Saquet et al. (2003) attributed the better tolerance of CA delayed fruit under stressful storage conditions to better membrane integrity and the balanced energy status of fruit. With pears, the browning disorder was attributed to the accumulation of reactive oxygen species (Franck et al., 2007), which reflects the significance of the antioxidants in preventing various types of storage disorders. In this sense, Watkins et al. (1997) found that postharvest treatment of 'Empire' apples with diphenylamine (DPA) prevented the development of external CO_2 injury in peel.

Accordingly, investigations to elucidate the mechanism by which the delay led to such a positive impact are needed, in particular at the molecular and cellular levels. Moreover, the interaction of ethylene with the delayed establishment of CA and external CO_2 injury also needs further investigation. In this respect, MacLean et al. (2006) suggested that 1-MCP, an inhibitor of ethylene action, may inhibit the biosynthesis of flavonoid compounds.

Literature about gene expression and 'skin burning' incidence with stored apples is absent. However, studies and findings in respect to another physiological disorder, superficial scald, may help in explaining the 'skin burning' disorder. In this respect, Whitaker (2004) reported that oxidative stress is the accepted cause of superficial scald. Since antioxidants may counteract oxidative stress in fruit, Huelin and Coggiola (1970) found that pre-storage treatment of apples with the antioxidant DPA largely controlled superficial scald development. With 'Cameo' apples, oxidative stress might be the cause for 'skin burning'. The reduction in the levels of several key polyphenols may deplete the fruits from their ability to counteract such stress. In this respect, Zubini et al. (2007) reported, with 'Granny Smith' apples, a positive correlation between H_2O_2 and levels of mRNAs for the antioxidant enzymes.

The molecular mechanisms of 'skin burning' by 'Cameo' apples still need further investigation. However, it is clear from our results that up-regulation in the expression of polyphenol-related genes in well-colored fruit was associated with the development of this disorder. Moreover, it is obvious that poorly colored and injured apples had lower transcript levels of *MdACS1* that reflects impairment in the biosynthesis of ethylene by these injured apples, which may further reduce the capacity of fruit to synthesize polyphenols. In this respect, De Wild et al. (2003) reported that ethylene production is inhibited by high CO_2 levels.

In conclusion, we have shown that 'skin burning' by 'Cameo' apples is related to the down-regulation of some polyphenol-related genes, in particular *MdCHS*, *MdCHI*, and *MdANS*. Moreover, proper lighting of fruit, through proper spacing and pruning of trees, proves to be crucial to get well-colored apples that can tolerate high CO_2 levels in storage. However, the exact mechanism of ethylene in respect to the expression of the genes assessed needs further investigation.

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