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Effects of epigallocatechin-3-gallate (EGCG) on skin greasiness and related gene expression in 'Jonagold' apple fruit during ambient storage

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ABSTRACT

Some apple (*Malus* × *domestica*) cultivars can become greasy during ripening due to changes in their surface wax composition. Epicuticular waxes are derived by the de novo synthesis of fatty acids, catalyzed by fatty acid synthase (FAS). Epigallocatechin-3-gallate (EGCG) has previously been shown to be an inhibitor of FAS in both animals and bacteria. Therefore, we hypothesize it may also affect the development of postharvest skin greasiness in apples. In this study, 'Jonagold' apples were treated with EGCG and then stored at 20 °C. The composition of the surface waxes was analyzed and the expressions of genes related to wax synthesis were recorded. The results show that at these postharvest storage temperatures, the level of skin greasiness rises rapidly. This is consistent with measured increases in the content of the main fluid waxes - linoleate and oleate esters. Compared with the controls, the accumulation of fluid waxes was suppressed by EGCG at most stages during ambient storage. A number of genes related to fluid wax biosynthesis, including *MdKASIII*, *MdKAR*, *MdFAD2* and *MdWSD11*, showed significant increases in expression during the development of skin greasiness. Meanwhile, the expressions of *MdKASII* and *MdENR*, the transport gene *MdLTPG1* and the regulation gene *MdSHN3* declined during storage. The expressions of all these genes were down-regulated by EGCG, while the fruit respiration and firmness were similar between the EGCG-treated and control fruit throughout the storage period. We suppose that the inhibitory effects of EGCG are to down-regulate expression levels of genes involved in wax biosynthesis. This results in reduced accumulation of the fluid wax components and delays and lowers the development of skin greasiness. Our results also indicate that *MdFAD2* and *MdWSD11* may be critical for formation of greasy esters in apple skins. This work is the first evaluation of the effects of EGCG on apple skin greasiness. The results supply useful information elucidating the mechanisms through which apple fruit skins can become greasy during postharvest storage.

1. Introduction

Like most aerial plant surfaces, the skins of apple fruit are covered with a hydrophobic cuticle whose principal function is to protect against desiccation. The cuticle contains cutin polyester matrix and intracuticular and epicuticular waxes (Jetter and Schäffer, 2001; Post-Beittenmiller, 1996). The epicuticular waxes of apple fruit consist of various long-chain fatty acids and their derivatives, alkanes, primary and secondary alcohols, aldehydes, ketones and esters (Kolattukudy, 1996; Kunst and Samuels, 2003). The cuticular waxes of some species may also include triterpenoids and sterols (Belding et al., 1998; Verardo et al., 2003; Veraverbeke et al., 2001).

It is well established that in apple the composition of cuticular waxes varies during development, and subsequent storage and shelf life (Bringe et al., 2006; Curry, 2008; Dong et al., 2012; Morice and Shorland, 1973; Veraverbeke et al., 2001). Greasiness is a physical and chemical fruit-surface phenomenon that can develop both on the tree and during storage and is associated with changes in the wax constituents (Curry, 2008; Dong et al., 2012; Morice and Shorland, 1973). Christeller and Roughan (2016) suggested that changes in the amounts and presence of novel long-chain unsaturated fatty acid esters of farnesol appear to be the major contributing components to 'Royal Gala' apple skin greasiness. Recent studies have revealed that it is the accumulation of more-fluid wax constituents that leads to the greasy feeling on 'Jonagold' and 'Cripps Pink' apples (Yang et al., 2017a,b). These more-fluid wax constituents consist mainly of linoleate and oleate esters of (E, E)-farnesol and short-chain alcohols (C3–C5). Fruit skin greasiness is among the most unappealing quality attributes of fresh fruit at the point of sale (Richardson-Harman et al., 1998). Treatments that inhibit or reduce the development of fruit skin greasiness may be of significant commercial value.

Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol

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in green tea, is an effective scavenger of reactive oxygen species in vitro and may also function as an antioxidant (Higdon and Frei, 2003; Wiseman et al., 1997). Previous studies have revealed that EGCG is an effective inhibitor of type I fatty acid synthesis (FAS) in humans and animals and of type II FAS in bacteria (Brusselmans et al., 2005; Puig et al., 2008; Wang and Tian, 2001; Zhang and Rock, 2004). The effect of EGCG on plant FASII is unknown. In plant, the FAS complex consist of Acetyl-CoA Carboxylase (ACC), Acyl-Carrier-Protein (ACP), Malonyl-CoA-ACP Transacylase (MCAT), β-Ketoacyl-ACP Synthase (KASI, KASII and KASIII), β-Ketoacyl-ACP Reductase (KAR), β-Hydroxyacyl-ACP Dehydratase (HAD) and Enoyl-ACP Reductase (ENR) (White et al., 2005). And this complex functions in the de novo synthesis of C16 and C18 fatty acids, part of the process resulting in the formation of the cuticular waxes (Kunst and Samuels, 2003; Kunst and Samuels, 2009; Lee and Suh, 2015; Samuels et al., 2008). Hence, a comprehensive study is worthwhile to extend our limited knowledge of the effects of EGCG on the changes in wax constituents during the development of apple skin greasiness.

In this work, we compared the changes in wax constituents and the expressions of key genes related to wax synthesis in EGCG treated 'Jonagold' apples stored at 20 °C. The main aim was to reveal any inhibiting effects of EGCG on skin greasiness in apple fruit during ambient storage.

2. Materials and methods

2.1. Plant materials and treatments

'Jonagold' apples (*Malus domestica* Borkh) were picked from the Baoji Qianyang apple experimental farm of Northwest A & F University, China, on 6 September 2016. Fruit were selected to be without visible damage and to be of uniform size. Fruit were delivered immediately to the laboratory and divided randomly into two groups. One group was treated for 15 min by immersion in 4.5 g L^{-1} EGCG (melonepharma, Dalian, China). The EGCG solution was made up by adding 22.5 g EGCG powder to 5 L of distilled water. The other group (control) was treated with distilled water. All fruit were air dried and then stored at 20 °C for 26 days by which time almost all the control fruit had become greasy.

2.2. Assessment of respiratory rate, fruit firmness and skin greasiness

The respiration rates of the fruit were measured regularly during storage with a CO_2 Infrared Gas Analyzer (Telaire 7001, Goleta, CA). Nine treated fruit and nine control fruit were selected at random and assigned as three replicate groups of three fruit and placed in three, 4 L glass jars. Each jar was sealed with a rubber plug for 60 min. Meanwhile, the gas analyzer readings were recorded every 20 min to calculate the final CO_2 concentration. Fruit firmness of nine fruit per group was measured regularly at opposite peeled sides with a GS-15 fruit texture analyzer (Guss Manufacture, Republic of South Africa) equipped with an 11 mm diameter probe. Peak forces are expressed in Newton. The skin tissues from each of the two groups of fruit were sliced into liquid nitrogen, and stored at −80 °C pending RNA preparation.

Skin greasiness was assessed quantitatively using the method described by Dadzie et al. (1995). Greasiness score was obtained by rubbing the fruit against the hand. The level of greasiness was assessed subjectively and semi-quantitatively at one of four levels: none (0), slight (1), moderate (2) or severe (3).

2.3. Cuticular wax extraction

Extraction of cuticular wax was carried out by immersing the fruit individually (n = 3 per treatment) three times in 400 mL of chloroform for 45 s. The solutions containing the dissolved waxes were combined, filtered, and concentrated in a rotary evaporator. After volume reduction, the wax concentrates were transferred to vials (50 mL) by adding 20 mL of chloroform/methanol (10:1, v:v). The samples were stored at −40 °C pending gas chromatography–mass spectrometry (GC–MS) analysis (Yang et al., 2017a,b).

2.4. Wax preparation and chemical analysis

After adding heptadecane as an internal standard, chloroform was evaporated under a gentle stream of nitrogen (N_2) at 40 °C. Wax mixtures were then treated with bis-N, *N*-(trimethylsilyl) trifluoroacetamide (BSTFA; AlfaAesar, Heysham, UK) for 45 min at 70 °C, converting the free hydroxyl and carboxyl groups into their trimethylsilyl ethers and esters (Wang et al., 2014; Yang et al., 2017b). After evaporating excess BSTFA under N_2, the samples were re-dissolved in chloroform for chemical composition analysis.

Qualitative analysis was carried out with a GC–MS (Thermo FisherScientific, Waltham, USA). A DB-35 MS capillary column (30 m, 0.32 mm i.d., 0.25 μm film, Agilent Technologies) was used for the separation of compounds using helium as carrier gas. A volume of 1 μL of each sample was injected into the column at 70 °C, held for 1 min; then increased to 200 °C at a rate of 10 °C min^{-1}; then increased to 300 °C at a rate of 5 °C min^{-1}, and held at 300 °C for 30 min. The MS was operated in the positive electron ionization mode at 70 eV, obtaining spectra with an *m/z* range of 45–650. Wax compounds were identified by retention index (RI) and by matching their electronionization mass spectra with those from the NIST 14 MS library.

Quantitative analyses were carried out using a FULI GC9790II gas chromatograph equipped with a flame ionization detector under the same conditions as GC–MS except that N_2 was used as the carrier gas. All compounds were quantified by peak area comparisons with the internal heptadecane and their relative amount are expressed per unit of fruit surface area. The fruit surface area was calculated according to Yuan et al. (1995).

2.5. Real-time quantitative RT-PCR analysis (qRT-PCR)

UBI, ubiquitin; LRR-PK1, Leu-rich repeat protein kinase1; KASII/III, β-ketoacyl-ACP synthaseII/III; KAR, β-ketoacyl-ACP reductase; ENR, enoyl-ACP reductase; FAD2, fatty acid desaturation enzyme2; WSD11, wax ester synthase11; LTPG1, glycosylphosphatidyl inositol-anchored lipid transfer protein1; SHN3: SHINE3.[a](Eccher et al., 2015), [b](Lashbrooke et al., 2015), [c](Albert et al., 2012) and [d](Yang et al., 2017a). The remaining sequences of these wax genes were obtained by searching the APPLE GENOME V1.0 predicted CDS with the proteins from Arabidopsis whose functions had previously been experimentally verified to be involved in wax synthesis. Primers for these genes were designed with Premier 5.0 software.

Total RNA was extracted using a plant RNA isolation kit (Omega Bio-tek, Norcross, GA, United States). The RNA concentration was evaluated with a Nanadrop 2000 and its quality was determined by running on a 1.0% agar ethidium bromide-stained gel. A quantified 1 μg aliquot of total RNA was reverse-transcribed to cDNA using the Prime Script RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Quantitative RT-PCR was carried out on an Icycler iQ5 (Bio-Rad, Berkeley, CA, USA) with the SYBR Premix Ex Taq II (TaKaRa, Dalian, China), in accordance with the manufacturer's instructions. Target gene expression was normalized to that of the internal reference genes *MdUBI* and *MdLRR-PK1* (Eccher et al., 2015) using the $2^{-\Delta\Delta CT}$ method. All reactions were run on three replicates for each biological repeat. All primers used in this study are listed in Table 1. And the phylogenetic tree analysis and conserved sequence alignment are presented in Supplementary material (Figs. S1–S5).