Postharvest Biology and Technology 145 (2018) 53–60

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Methyl ester generation associated with flesh browning in 'Fuji' apples after long storage under repressed ethylene function

Fukuyo Tanaka[a,*], Miho Tatsuki[b], Kazuya Matsubara[c], Keiki Okazaki[a], Masatoshi Yoshimura[d,e], Satoshi Kasai[f]

[a] Central Region Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, 305-8666, Japan
[b] Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO), 2-1 Fujimoto, Tsukuba, Ibaraki, 305-8605, Japan
[c] Ritsumeikan University, 1-1-1, Noji-higashi, Kusatsu, Shiga, 525-8577, Japan
[d] Food Research Institute, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, 305-8642, Japan
[e] Tokyo University, 1-1-1, Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan
[f] Apple Research Institute, Aomori Prefectural Industrial Technology Research Center, Botandaira, Kuroishi, Aomori, 036-0332, Japan

ARTICLE INFO

Keywords:
Apple flesh browning
Methyl esters
Lipid membrane integrity
PME
Bio-marker screening
CA storage

ABSTRACT

'Fuji' apples stored under depressed ethylene by 1-MCP treatment (1-MCP) or in CA storages with 1.5% or 5.0% CO_2 (CAL or CAH) were profiled for volatile production related to flesh browning severity after 4, 6 and 8 months of storage. Among the volatiles observed, methyl esters correlated with browning area at the developing stage. This relationship was further demonstrated using apples harvested the following year. Expression levels of genes related to senescence and alcohol synthesis were compared and expression of *MdPME2* tended to be higher in injured apples. However, methyl esters increased with *MdPME2* levels only in healthy individuals, suggesting that pectin methyl esterase (PME) may regulate methanol liberation from pectin well before visible browning development. Thus, methyl ester generation reflected flesh browning of 'Fuji' apples, which may be associated with PME activation that induces methanol liberation and oxidative stress after long storage.

1. Introduction

'Fuji' is one of the most popular apple cultivars in the world, due to their high quality, sweet flavor, and long shelf life (Abbott et al., 2004; Bonany et al., 2014, 2013; Harker et al., 2008), however, 'Fuji' also are highly susceptible to internal flesh browning after long periods of controlled atmosphere (CA) storage (Amiot et al., 1992; Argenta et al., 2002b; Kasai and Arakawa, 2010; Matsubara et al., 2017; Volz et al., 1998a). Similarly, storage under anaerobic conditions induced disorders, with a high CO_2 environment and watercore enhancing symptoms (Argenta et al., 2002a; Harker et al., 2008; Kasai and Arakawa, 2010).

In addition to storage environments, volatiles, including acetaldehyde, ethyl esters, and methyl esters, can further accelerate browning (Argenta et al., 2002a, b; Argenta et al., 2004; Lumpkin et al., 2015; Volz et al., 1998a). Although energy- and lipid-related processes, including an important role for oxidative stress, have been shown to accompany browning (Franck et al., 2007; Hatoum et al., 2016; Ho et al., 2013; Kasai and Arakawa, 2010; Mellidou et al., 2014), the physiological mechanisms and metabolic changes underlying the symptoms have not been elucidated.

In general, flesh browning is caused by an oxidative reaction where polyphenols are catalyzed mainly by polyphenol oxidase (PPO) and phenol peroxidase (POD) (de Castro et al., 2008; Toivonen, 2004; Toivonen and Brummell, 2008). Most polyphenols are located in the vacuole, with the remnant occur in the apoplast/cell wall (Toivonen, 2004). Because PPO and POD are located in plastids and the cytosol, respectively, the breakdown of membranes is required for browning, which is a result of the direct interaction of polyphenols and enzymes. The decaying of the cell membrane, tonoplast, and plasmalemma result in metabolic changes that are key to understanding apple browning. However, the specific biochemical reaction underlying flesh browning, and whether it is due to polyphenols or the involvement of membrane integrity, are unknown (Mellidou et al., 2014).

In this study, we aimed to propose volatile bio-markers for the development of flesh browning in 'Fuji' apples using non-destructive analyses. We analyzed a number of apples individually using newly applied volatile-trapping system contrasting with the severity of flesh browning to examine the biochemical mechanisms responsible for symptom progression. We also aimed to elucidate the potential

* Corresponding author.
E-mail address: fukuyot@affrc.go.jp (F. Tanaka).

https://doi.org/10.1016/j.postharvbio.2018.06.002
Received 29 March 2018; Received in revised form 1 June 2018; Accepted 15 June 2018

mechanisms of flesh browning after long-term storage under repressed ethylene function. We then analyzed expression levels of genes related to the synthesis of browning bio-markers, such as alcohol generation and senescence. We conclude by discussing the involvement of methanol, and its liberation from pectin by pectin methyl esterase (PME), to associate bio-markers with the reaction of polyphenol oxidation.

2. Materials and methods

2.1. Fruit materials

'Fuji' apples were harvested at their commercial ripening stage on 4 November 2014 from the experimental orchard of the Apple Research Institute in Aomori, Japan. Harvested fruit were divided into four experimental treatments: control (CON), 1-methylcyclopropene (1-MCP), CA low CO_2 (CAL) then CA high CO_2 (CAH). For 1-MCP, apples were treated for 24 h at room temperature with 1 µl/l of 1-MCP using SmartFresh™ (Agrofresh Inc., Philadelphia, PA, USA). After the treatment, fruit of 1-MCP and CON treatments were stored at 0 °C under ambient air conditions. In CAH and CAL, fruit were maintained in ambient air conditions at 0 °C for three weeks after harvest, then stored at 0 °C under different CO_2 conditions (CAH: 5.0% CO_2, 2.2% O_2; CAL: 1.5% CO_2, 2.2% O_2). Atmospheric conditions in a tightly sealed refrigeration room with a volume of 23.8 m^3 were maintained using a CA system (Fujiplant Co., Ltd., Aomori, Japan), which automatically controlled atmospheric concentrations within 0.1% of target values. The apples were removed from storage 4, 6, and 8 months after harvest. Ten apples were sampled at random in each treatment. At the 6-month period, to secure a sufficient number of injured apples and screening performance, several apples, which were predicted to develop browning by fruit-sorting machine, were selected and added to the10 randomly selected samples. On the day the samples were shipped at 4 °C to the National Agricultural Research Organization (NARO) in Ibaraki. After arrival, they were placed at 20 °C overnight and used for analysis.

We repeated the experiment a second time the following year, 2015. Apples were harvested in the same orchard as in 2014 at commercial ripening stage. Fruit were harvested from two different plots (A and B) and pooled. Here, Plot B apples were harvested from an area with better photoenvironment conditions than those for Plot A apples. They were maintained under ambient air conditions at 0 °C for three weeks, and then stored under controlled atmospheric conditions (1.5% CO_2, 2.2% O_2 at 0 °C, CAL in 2014). After 5 months, fruit were removed from CA storage and maintained under ambient air conditions at 0 °C. After 6 months of storage, 40 fruit a plot were removed from storage and shipped at 4 °C to NARO on the same day. They were stored at 4 °C until assessment. To decide the appropriate time for validation, requiring browning assessment with destructive analysis, we performed destructive monitoring using few samples three times. Five of each replicate were utilized for destructive monitoring, and the remaining 70 fruit were used for actual validation. Subsequently, relevant gene expressions for the selected samples from Plot A were analyzed.

2.2. Rating of the browning severity

Each apple was cut horizontally at the equator, and the section was captured by a flatbed image scanner (GT-X980; Epson, Suwa city, Japan). The cover of the transparency unit was removed to accommodate the fruit. To eliminate external lighting, the fruit were covered by a black Bakelite board during scanning. The scanning resolution was 1200 dpi and the color depth was 16 bits per R/G/B component (Matsubara et al., 2017). To rate the browning severity, we employed subjective evaluation by an expert who has been studying apple browning for years. The expert estimated percent browning area of flesh by viewing images on a liquid-crystal monitor.

Table 1
Oligonucleotide primers used in real-time qRT-PCR.

Cone name		oligonucleotide sequence
MdPME2	F	AGCTCACTAAGCGTGAGAAG
	R	TACTCATGGAGATCCTCGAC
MdPDC1-2	F	GGTAACTGCACAGGACATTT
	R	GTAGCCTCCGTTGTTGATAA
MdADH1-1	F	CAGTTCAAGAGGTGATTGCT
	R	CAGTGCTTCCTGTACATTCA
MdADH1-2	F	CCGTTCAAGAGGTGATAGCT
	R	CAACATTTCCTGTGCATTCA
MdADH2-1	F	ACTTTGCCACACTGATCTCT
	R	AACACTCTCAACGATTCCAG
MdPG1	F	CACCGGTGGGATAGCAACAT
	R	TCCAAAATCGTCGACGCTAAT
MdACS1	F	GCCTTCCGGGTTTTCGA
	R	GGCGGCCACAACCATGT
MdACS3	F	GGAAAAGCACTTGGAAGAAAATTC
	R	CGCTCCTTTTGATCCCTTTG
MdACO1	F	GAATGTCGATAGCCTCGTTCTACA
	R	GGTGCTGGGCTGATGAATG
Actin	F	GATTCCGGTGCCCAGAAGT
	R	CCAGCAGCTTCCATTCCAA

2.3. Validation of browning responses to candidate markers and real-time quantitative RT-PCR

Apples from the 2015 experiment, which were stored at 4 °C after CAL storage, were cut horizontally at the equator. The surfaces of the upper halves were scanned for rating of the browning severity. The lower halves were cut again horizontally at the equator to 1 cm thickness, and then several disks were obtained from the flesh using a cork borer. The flesh disks were maintained at −80 °C until RNA was isolated. For fruit that developed browning, sites neighboring injured areas were used, because brown tissue is a group of dead cells (de Castro et al., 2008), which is difficult to obtain quality RNA. Four injured and four healthy apples were selected based on browning severity and generation of methyl esters, which were candidate bio-markers for browning. The target genes were enzymes related to methanol or ethanol synthesis, maturing, and senescence, and included PME, pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), 1-aminocyclopropanecarboxlic acid (ACC) synthetase (ACS), ACC oxidase (ACO), polygalacturonase (PG) (Table 1).

We used real-time quantitative RT-PCR (qRT-PCR) to assess expression levels of the relevant genes. Detailed protocols were described in a previous paper (Tatsuki et al., 2011), wherein the total RNA of flesh disks was extracted from frozen samples by hot borate method (Wan and Wilkins, 1994), using DNase I (TURBO DNA-free Kit, Ambion, Austin, CA, USA). First-strand cDNA was prepared with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems) with a SYBR Premix Ex Taq kit (TaKaRa, Kyoto, Japan), using a set of primers designed using Primer Express software (Applied Biosystems) for each of nine amplified genes (Table 1). Actin mRNA was used as an internal standard in all experiments. qRT-PCR was performed at least twice for each gene using aliquots of the same purified total RNA, but one dataset was used to produce the figures. The mean of three individual PCR experiments was determined from separate, but concurrent reactions.

2.4. Analysis of volatiles from apple using GC/MS

2.4.1. Static trapping of apple volatiles

To enable sharing of the same fruit across all non-destructive analyses in 2014 (data were not used in this study), and also to repeat analyses of identical individuals in 2015, we used a non-destructive method to measure gas emissions from the head space from whole