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Abscisic acid is involved in aromatic ester biosynthesis related with ethylene in green apples

Shanshan Wang[a,1], Takanori Saito[a,1], Katsuya Ohkawa[a], Hitoshi Ohara[a,b], Sirinan Suktawee[a], Hiromi Ikeura[c], Satoru Kondo[a,*]

[a] Graduate School of Horticulture, Chiba University, Matsudo 271-8510, Japan
[b] Center for Environment, Health and Field Sciences, Chiba University, Kashiwa-no-ha 277-0882, Japan
[c] Faculty of Life and Environmental Science, Shimane University, Matsue 690-8504, Japan

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ABSTRACT

The production of aromatic volatiles such as esters during the ripening process in climacteric fruits is known to be controlled by ethylene. However, we here show that abscisic acid (ABA) application accelerated the onset of short-chain ester production (hexyl propionate, ethyl-2-methyl butyrate) and the expression of biosynthesis genes (*MdAAT2* and *MdBCAT1*) during ripening of 'Orin' apple. ABA application also promoted the production of ethylene, and caused ethylene peak shifts correlated with the expression of ethylene synthesis genes (*MdACS1/3* and *MdACO1*), suggesting that ABA may act jointly with ethylene as a positive regulator at the ripening stage of 'Orin' apple. Additionally, endogenous levels and expression of biosynthesis (*MdNCED1*) and signal transduction genes (*MdABF2*-like) of ABA increased towards ripening. Finally, the localization of the putative MdABF2-like protein binding element, AREB/ABF, was observed in the 5′-upstream region of *MdACS1/3* and *MdACO1*.

1. Introduction

The ripening process of the apple (*Malus* × *domestica* Borkh.), involves numerous and complex chain reactions, such as physiological changes in hormone levels, enzyme activity, and respiration rate. These changes quickly induce texture softening, change in peel color, an accumulation of sugars, and a production of aromatic volatiles (Costa et al., 2012). The aromatic volatiles, including alcohols, aldehydes, ketones, sesquiterpenes, polypropanoids, and esters, are known as the important traits that attract consumers (Dimick et al., 1983). The apple is considered as a climacteric fruit, whose changes in emissions of aromatic volatiles quickly take place during the ripening stages. A previous study demonstrated that compounds of esters and alcohols, especially the fruity esters (e.g., butyl acetate, hexyl hexanoate, hexyl propanoate, 2 methyl butyl acetate, and ethyl-2-methyl butanoate), were produced toward the ripening stage, and contributed to the potent aromatic characteristics typical of the apple flavor (Dixon and Hewett, 2000). The fruity esters are primarily metabolized via two biosynthetic pathways, the fatty acid and amino acid pathways (Fig. S1) (Mathews and Van Holde, 1996; Wyllie and Fellman, 2000). In apples, straight chain esters are synthesized from lipids that are broken down initially through the β-oxidation of fatty acids by lipoxygenase (LOX) activity (Rowan et al., 1999). Sanz et al. (1997) reported that β-oxidation of fatty acids is the primary biosynthetic process providing acyl coenzyme A (CoAs) for ester formation. Acyl CoAs are reduced to aldehydes, which are in turn reduced to alcohols, by alcohol dehydrogenase (ADH), for use by alcohol acyltransferase (AAT) to produce esters (Bartley et al., 1985). Branched chain esters are produced from leucine, isoleucine, and valine by the amino acid pathway (Rowan et al., 1996). These amino acids can also be the precursors of acyl CoAs, which were catalyzed by branched chain amino acid aminotransferase (BCAT).

In climacteric fruits, ethylene has been widely accepted as the phytohormone regulating biochemical and physiological changes, including firmness, peel color, and flavor (Lelièvre et al., 1997). Ethylene is synthesized from S-adenosyl-methionine by the action of the enzymes 1-aminocyclopropane-1-carboxylic acid synthase (ACS, EC 4.4.1.14) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO, EC 1.4.3) (Bleecker and Kende, 2000). 1- Methylcyclopropene (1-MCP), as an ethylene inhibitor, is considered to bind to metals in ethylene receptors and prevent ethylene binding in its action (Sisler and Serek, 1997). The relationship between ethylene and the production of aromatic volatiles has been revealed through the application of both ethylene and 1-MCP, which leads to changes in levels of esters in apples (Yang et al., 2016). Moreover, they identified that the expression pattern of 17 genes

* Corresponding author.
E-mail address: s-kondo@faculty.chiba-u.jp (S. Kondo).
[1] These authors contributed equally to this work.

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related to the biosynthesis of the aromatic volatiles of the apple fruit, including *MdAATs*, *MdBCATs*, and *MdADHs*, fluctuated in response to ethylene and 1-MCP treatments.Abscisic acid (ABA) is well known for its pivotal roles in seed maturation, germination, and responses to environmental stresses (Finkelstein et al., 2002; Fujii and Zhu, 2009). At the same time, it has also been highlighted that it may be involved in the regulation of ripening in non-climacteric fruits such as strawberries and grapes, in which 9-*cis*-epoxycarotenoid dioxygenase genes (*NCED*), encoding a key ABA synthesis pathway, are up-regulated during the ripening stage (Jia et al., 2011). In fact, ABA was also observed to promote sugar metabolism and accumulation in citrus and grape berry (Wang et al., 2007; Pan et al., 2005) The ABA signaling pathways model assumed that the pyrabactin resistance 1-like/2C-type protein phosphatases/SNF1-related kinase 2 signaling network (PYR1-PP2C-SnRK2), in which ABA promotes the interaction of PYR1 and PP2C, resulted in PP2C inhibition and SnRK2 activation. This transduced the ABA signal through the phosphorylation of downstream factors such as abscisic acid response element-binding/abscisic acid response element-binding factor (AREB/ABF) (Fujii and Zhu, 2009; Ma et al., 2009; Park et al., 2009). In the grape, *VvABF2* has been proven to be involved in several ABA-mediated ripening-related pathways, positively regulating the grape fruit ripening process (Nicolas et al., 2014). Recently, in climacteric fruits, the possibility of the involvement of ABA in fruit ripening was suggested. A transgenic study using the tomato showed that the overexpression of *SlAREB1* resulted in increasing organic acids, hexoses, and amino acids in immature, mature green, and red ripe tomatoes, suggesting the *SlAREB1* participates in the regulation of metabolic programming that takes place during fruit ripening (Bastías et al., 2014). In Rosaceae species, the exogenous ABA treatment involved in the stimulation of fruit ripening was also reported in the peach (Soto et al., 2013). The relationship between fruit ripening of climacteric fruits in Rosaceae species and ABA was partially explained by the effect of ethylene production; however, the genetic mechanism is still unclear.

The crosstalk between ethylene and ABA is well discussed in seed germination and dormancy. In the seeds of Arabidopsis, the interaction between ethylene and ABA was thought to modulate sugar metabolism during the early stages of seed germination (Dekkers et al., 2008). A pioneer study using transgenic Arabidopsis revealed that the ABA hypersensitive mutant (*enhanced response to ABA3*) was identified as an ethylene signaling mutant, *ETHYLENE INSENSITIVE2* (Ghassemian et al., 2000). Similarly, *the enhancer of abi1* mutant, was also identified as the ethylene response mutant, *CONSTITUTIVE TRIPLE RESPONSE1*, suggesting that ethylene negatively regulated ABA signaling in seed germination (Beaudoin et al., 2000). In contrast, during fruit ripening in tomatoes, the expression and accumulation of *LeNCED1* and ABA content were indicated prior to that of *LeACS2/4* and *LeACO1* (Zhang et al., 2009). They also confirmed that the treatment of the ABA inhibitor, nordihydroguaiaretic acid, delayed fruit ripening and softening, assuming ABA acts as a positive regulator of ethylene in the ripening of the tomato fruit. In citrus, it was previously reported that the transcripts of *CsACO1* in ABA-treated fruits were significantly higher than that in untreated fruits during the late ripening stages (Wang et al., 2016). Furthermore, the expression of both, ethylene signaling (*ETR1/2*) and biosynthesis (*ACS1* and *ACO1*) genes was up-regulated by ABA application in the peach (Soto et al., 2013). Collectively, it could be assumed that ethylene and ABA were both implicated in the complex fruit ripening process, and their mechanisms could not act independently in the ripening of fruits.

In this study, to clarify the complex relationship between ABA and ethylene during apple ripening, we first evaluated the effects of exogenous ABA and 1-MCP applications on changes in peel color, total soluble solids (TSS), aroma volatiles, and related genes during the ripening of the green apple variety, 'Orin'. We found that the ripening process was slightly strengthened with the ABA treatment; therefore, we evaluated the effects of ABA application on ethylene and ethylene biosynthesis genes (*MdACS1/3a* and *MdACO1*). Finally, since we could reveal the possibility that the ethylene biosynthesis genes were included as a member of the AREB/ABF regulon, the fluctuations in ABA content, the expression of *MdNCED1* and *MdABF2*-like genes, and the presence of the ABF-binding site in the 5′-upstream region of ethylene biosynthesis genes were investigated.

2. Materials and methods

2.1. Plant materials and treatments

The green variety 'Orin' apples (*Malus* × *domestica* Borkh.) of uniform size and peel color were harvested from the orchard in the Center for Environment, Health, and Field Sciences, Chiba University, which is located in Numata, Gunma, Japan (37°N, 139°E), 160 days after full bloom in 2015. The fruits were divided into three groups, two groups were subjected to the exogenous treatments of S-ABA (1.9 mM, Wako, Osaka, Japan) and 1-MCP (1.0 ppm, smart fresh 0.14%, Agrofresh, PA, USA) and the third was the untreated control group. Prior to its application, S-ABA was dispersed in water with 0.1% (v/v) of a surfactant (Approach BI, 50% polyoxyethylene hexitan fatty acid ester; Kao, Osaka, Japan) and then the fruits were immersed in solution for 5 min. 1-MCP was applied to the treated group in a sealed container for 12 h at 20 °C. The untreated control fruits were immersed in distill water contained 0.1% (v/v) Approach BI for 5 min. After treatment, all fruits were air-dried and kept in a controlled room at 25 °C with 95% relative humidity. Nine apples (one replication of three fruits) from each treatment were sampled at 9, 16, and 23 days, respectively. On each sampling day, after ethylene production was analyzed, peel was frozen with liquid N_2 and stored at −80 °C immediately.

2.2. Total soluble solid (TSS) and chlorophyll content

Juice was squeezed from the pulp of apples for measuring TSS with a conventional refractometer (three replications per treatment). The method used for the chlorophyll analysis was designed based on previous research (Khaleghi et al., 2012; Hu et al., 2013). In brief, 0.5 g of pigment was extracted by immersing in the extraction buffer (80% acetone and 20% (v/v) 0.2 M Tris-HCl pH 8.0) for 24 h at 4 °C in the dark, until completely colorless. The absorbance of the chlorophyll extractions was measured at 645 and 663 nm, using a spectrophotometer. The total chlorophyll content was calculated with Arnon's equation (Khaleghi et al., 2012).

2.3. Prime esters compound analysis

Ester volatile compounds were extracted and identified by gas chromatography-mass spectrometry (GC–MS) based on our previous research, then we developed the quantification by gas chromatography with flame ionization detection (GC-FID) (Wang et al., 2015). The extraction of aromatic volatiles was performed in three replications for each treatment using solid phase micro extraction (SPME); the frozen samples (1.0 g) were sealed in a 4-mL vial with 1.0 μg of cyclohexanol as an internal standard, then the headspace gases were extracted for 30 min at 40 °C using SPME with 65-μM polydimethylsiloxane-divinylbenzene fiber (Supelco, Bellefonte, PA). Subsequently, samples were directly desorbed at the injection port of GC–MS (QP-5000, Shimadzu, Kyoto, Japan) and GC-FID (GC-4000 plus, GL Sciences, Kyoto, Japan), which was set at 250 °C. A DB-WAX column (60 m × 0.25 mm ID × 0.25-μM film thickness; Agilent, Santa Clara, CA) with a helium gas linear flow rate of 30 cm/min used to separate the aromatic volatiles. The column temperature was isothermal at 40 °C for 7 min, then was increased by 5 °C/min to 220 °C, and finally increased by 20 °C/min to 245 °C and maintained there for 15 min. The compounds were identified by comparing the spectra in the National Institute for Standards and Technology (NIST 21 and 27) library and the mass spectra