Opposite Accumulation Patterns of Two Glycoside Hydrolase Family 3 α-L-Arabinofuranosidase Proteins in Avocado Fruit during Ripening

Yusuke Kamiyoshihara¹, Shinji Mizuno¹, Mirai Azuma¹, Fumika Miyohashi², Makoto Yoshida², Junko Matsuno³, Sho Takahashi⁴, Shin Abe¹,⁴, Hajime Shiba¹,⁴, Keiichi Watanabe¹,⁴, Hiroaki Inoue¹,⁴ and Akira Tateishi¹,⁴*

¹College of Bioresource Sciences, Nihon University, Fujisawa 252-0880, Japan
²Kanagawa Agricultural Technology Center, Hiratsuka 259-1204, Japan
³Aichi Agricultural Research Center, Nagakute 480-1193, Japan
⁴Graduate School of Bioresource Sciences, Nihon University, Fujisawa 252-0880, Japan

Avocado fruit ripen with ethylene production after harvest and the flesh becomes soft and edible due to degradation of cell wall polysaccharides during ripening. α-L-Arabinofuranosidase is a hydrolytic enzyme known to digest arabinose-containing cell wall polysaccharides. It has been shown that its activity increased with fruit ripening. However, our previous study showed that an α-L-arabinofuranosidase gene (PaArf/Xyl3A) is expressed in the avocado fruit before ethylene production. In addition, the transcripts were detected in some organs in which the level of ethylene was extremely low. These results indicate that the gene expression is independent of ethylene. In the present study, we carried out immunoblot analyses of α-L-arabinofuranosidase at the protein level. Using a polyclonal antibody raised against Japanese pear α-L-arabinofuranosidase, two α-L-arabinofuranosidase proteins with molecular masses of 72 kDa and 68 kDa, presumably belonging to glycoside hydrolase family 3, were detected in ripening avocado fruit. The protein levels in ethylene or 1-methylcyclopropene (1-MCP)-treated fruits were examined and the results indicated that the two proteins responded to ethylene in opposite ways; the 68 kDa protein showed a temporary accumulation, whereas the 72 kDa protein exhibited dissipation possibly caused by a loss of stability. The total enzyme activity of α-L-arabinofuranosidase was elevated faster in the ethylene-treated fruit throughout ripening and was slower in the 1-MCP-treated fruit, suggesting the existence of another α-L-arabinofuranosidase, which did not cross-react with the antibody and was positively regulated by ethylene, in ripening avocado fruit.

Key Words: arabinan, arabinose, cell wall, 1-MCP.

Introduction

Ethylene is a plant hormone that influences various aspects of plant growth including fruit ripening. Its signal triggers the expression of a wide range of ripening-related genes, inducing fruit ripening with dramatic changes such as starch degradation, reduction in organic acids, aroma evolution, change in color, and flesh softening (Gray et al., 1992; Seymour et al., 1993). Since fruits become edible after flesh softening caused by cell wall degradation, several cell wall modifying enzymes have been discussed in relation to the ethylene signal. α-L-Arabinofuranosidase is an enzyme responsible for releasing arabinosyl residues from cell wall polysaccharides and increases in activity with fruit ripening have been observed in many fruit species (Brummell, 2006). The structural changes in arabinose-containing polysaccharides seem to contribute to textural changes in fruit flesh (Brummell et al., 2004; Nara et al., 2001; Nobile et al., 2011; Orfila et al., 2001; Peña and Carpita, 2004). α-L-Arabinofuranosidase is also involved in modifications of cell wall polysaccharides in developmental processes throughout plant growth, including textural changes in fruit flesh during ripening.

α-L-Arabinofuranosidases in higher plants are divided into two glycoside hydrolase (GH) families...
(Cantarel et al., 2009; Davies et al., 2005), GH family 3 and 51. The members in the families show characteristic gene expression patterns and differential enzymatic properties (Minic and Jouanin, 2006; Tateishi, 2008). Since many ripening-related genes are induced by the ethylene signal, the effect of ethylene treatment on α-L-arabinofuranosidase expression, particularly at the transcriptional level, has been extensively studied (Hayama et al., 2011; Mwaniki et al., 2007; Storch et al., 2015; Yoshioka et al., 2010). In many cases, however, it appears that the gene expression is not specifically regulated by ethylene or a ripening signal. For example, three family 51 α-L-arabinofuranosidase genes (FaAra1-3) have been isolated from non-climacteric strawberry fruit (Fragaria × ananassa) and their gene expressions increased during ripening, but the transcripts could be also detected in immature fruit (Rosli et al., 2009). Although ethylene-dependent increases in expression of a family 51 α-L-arabinofuranosidase gene (PcARF1) have been observed in both European and Chinese pears (Pyrus communis and P. bretschneideri, respectively), the transcripts were also detected in the fruit before ripening (Mwaniki et al., 2007). As for family 3 α-L-arabinofuranosidase, PpARF/XYL from peach (Prunus persica) fruit has been shown to be up-regulated during ripening and a higher concentration of ethylene promoted elevation of the transcript level (Di Santo et al., 2009; Hayama et al., 2006), but the gene was also expressed in the vegetative organs in which ethylene production was barely detectable (Di Santo et al., 2009). Similarly, the transcript of the apple (Malus domestica) family 3 α-L-arabinofuranosidase gene, MdAF3, which associates with mealy texture formation of fruit flesh during ripening, was also found at the early ripening stage (Nobile et al., 2011). Taken together, it is likely that expression of α-L-arabinofuranosidase genes in fruits is quantitatively enhanced by either ethylene or a ripening signal; those signals are not absolute regulators of gene expression.

Avocado (Persea americana Mill. ‘Fuerte’) fruit were harvested from Yamada Orchard, Numazu, Shizuoka prefecture in Japan. The fruit were exposed to either 1000 μL·L⁻¹ of ethylene or 10 μL·L⁻¹ 1-methylocyclopropene (1-MCP) in desiccators at 15°C for 3 days and 1 day, respectively (Tateishi et al., 2007). The control fruit were also closed up in desiccators without ethylene or 1-MCP. The gases in the desiccators were exchanged every day. About 100 mL of potassium hydroxide solution was also placed in each desiccator to absorb carbon dioxide emitted from the fruit. After the treatments, the fruit were taken from the desiccators and ripened at 20°C. Five fruits were randomly sampled from each treatment and individual fruit were closed up in desiccator again. Ethylene concentrations in the headspace of the desiccators were measured by a gas chromatograph (GC-14A; Shimadzu, Japan) equipped with a flame ionization detector with a Porapak Q column (310 × 0.32 cm, 50/80 mesh, Shinwa Chemical Industries, Japan). Then the fruit mesocarp was sliced and frozen in liquid nitrogen, and stored at −85°C until use in further experiments.

**Protein extraction**

Fruit tissue was homogenized on ice in 100 mM potassium phosphate buffer (pH 6.0) containing 1.0 M sodium chloride, 30 mM 2-mercaptoethanol, and 0.1% (w/v) sodium ascorbate. Cell wall bound proteins were solubilized by stirring gently for 1 h at 4°C. After centrifugation (20000 × g, 20 min, 4°C), the supernatant was recovered, passed through four layers of cheesecloth and then dialyzed against 10 mM potassium phosphate buffer (pH 6.0) containing 10 mM sodium chloride and 10 mM 2-mercaptoethanol overnight at 4°C. The dialysis buffer was replaced two times. The crude protein extracts were used for immunoblot analysis and enzymatic activity assay.

**Immunoblot analysis**

Protein separation was performed according to a standard SDS-polyacrylamide gel electrophoresis method described by Laemmli (1970). After electrophoresis, the proteins in the gel were electro-blotted onto a polyvinylidene difluoride membrane using semi dry blotting apparatus (Trans-Blot SD Semi-Dry Transfer cell; BioRad Laboratories, USA). The membrane was blocked in 1% blocking reagent (Roche Diagnostics, Germany)
in Tris-buffered saline buffer. The blocked membrane was incubated for at least 1 h with a 1000-fold dilution of an anti α-L-arabinofuranosidase polyclonal antibody (Tateishi et al., 2005) in the same buffer. After several washings, the membrane was incubated with an anti-rabbit IgG-alkaline phosphatase-conjugated goat antibody. The signal was visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development after the washing steps.

Measurement of α-L-arabinofuranosidase activity
A reaction mixture (0.4 mL) containing 100 mM sodium acetate buffer (pH 4.5), 1.2 mM 4-nitrophenyl α-L-arabinofuranoside (Sigma-Aldrich, USA), and enzyme solution was incubated for 60 min at 34°C. The reaction was terminated by addition of 0.6 mL of 0.2 M sodium carbonate. The amount of the released 4-nitrophenol was measured by a spectrophotometer at 400 nm.

Results and Discussion

Immuno-detection of α-L-arabinofuranosidases in ripening avocado fruit
To detect α-L-arabinofuranosidases in avocado fruit, we performed immunoblot analyses using a polyclonal antibody raised against α-L-arabinofuranosidase derived from Japanese pear (Tateishi et al., 2005). The antibody cross-reacted with two proteins with molecular masses of 68 kDa and 72 kDa, respectively (Fig. 1). Neither protein was detected with the pre-immune serum and no other cross-reacted bands were detected (data not shown). Molecular masses of the cross-reacted proteins matched well with the mature forms of glycoside hydrolase family 3 α-L-arabinofuranosidase proteins from other plant species, including the Japanese pear (Fig. 1, Lane C, Tateishi et al., 2005). Although the theoretical molecular masses of family 3 α-L-arabinofuranosidases are approximately 80 kDa based on the deduced amino acid sequences including PaArf/Xyl3 from avocado (Fig. 2), the masses of the mature forms were less than that. The reduction could be due to post-translational processing of the protein (Lee et al., 2003) or an alternative splicing of the gene (Di Santo et al., 2015). In either case, the truncated forms of α-L-arabinofuranosidase that cover the entire catalytic domain are functional (Di Santo et al., 2015).

α-L-Arabinofuranosidase in higher plants constitutes a small gene family. An alignment of deduced amino acid sequences of α-L-arabinofuranosidase derived from avocado (PaArf/Xyl3A) and the Japanese pear (PpArf2) is shown in Figure 2. About 70% of amino acid residues of PaArf/Xyl3A and the Japanese pear were identical with that of PpArf2. In our previous study, Southern blot analysis revealed that there are at least two similar α-L-arabinofuranosidase genes in the avocado genome (Tateishi et al., 2015). Based on these results, it is most likely that the two proteins detected by the antibody were avocado α-L-arabinofuranosidases.

Fig. 1. Immuno-detection of two α-L-arabinofuranosidase proteins.
The fruit were ripened at 20°C. Five fruit were sampled on the indicated day and total proteins were extracted from the fruit and separated by SDS-PAGE. The proteins were electroblotted onto a PVDF membrane and subjected to immunoblot analysis. A polyclonal antibody raised against the Japanese pear α-L-arabinofuranosidase (PpArf2) was used. Two bands were detected (indicated arrows). The band in Lane M is a molecular marker (albumin, 66 kDa). The number in each lane indicates days after air treatment (control fruit) and in Lane C the loaded crude protein extraction from Japanese pear fruit (positive control, 62 kDa).

Fig. 2. Alignment of deduced amino acid sequences α-L-arabinofuranosidase derived from avocado (PaArf/Xyl3, BAQ19511.1, upper line) and the Japanese pear (PpArf2, BAD9523.1, lower line) using the Clustal W program (Thompson et al., 1994). The signal peptide experimentally confirmed is removed from PpArf2 sequence (Tateishi et al., 2005). An aspartic acid residue (position 293) in each line, which is a putative catalytic active site, is squared. Conserved amino acid residues between avocado and the Japanese pear are indicated with asterisks.
Distinct accumulation pattern of two α-L-arabinofuranosidase proteins during avocado fruit ripening

As shown in Figure 1, the detected α-L-arabinofuranosidases proteins derived from avocado exhibits distinct accumulation patterns. In the air-treated control, the level of the 72 kDa protein (the upper band) was low right after harvest. It showed an increase at 2 and 4 days after air treatment and then decreased thereafter. Concerning the endogenous ethylene production, it peaked at 6 days after treatment (DAT) in the control fruit (Fig. 3). It is clear that the level of the 72 kDa protein decreased gradually after the peak of ethylene production. The 68 kDa protein (the lower band) was also detected in the fruit right after harvest. The level was higher than that of the 72 kDa protein (Fig. 1). It showed an increase at 2 and 4 DATs and reached a peak at 6 DAT followed by a slight decrease at 8 and 10 DATs. Our results clearly showed that the two α-L-arabinofuranosidases proteins exhibited distinct accumulation patterns during ripening.

Effect of ethylene on accumulation of α-L-arabinofuranosidase proteins

To elucidate the relationship between ethylene production and the accumulation patterns of the α-L-arabinofuranosidase proteins, the fruit were treated with either exogenous ethylene or 1-MCP, a potent inhibitor of ethylene action (Sisler and Serek, 1997). As shown in Figure 4, exogenous ethylene treatment promoted endogenous ethylene production compared to the control fruit (Fig. 3). While the ethylene production was extremely low in the 2 DAT fruit, the maximum production was observed in 4 DAT fruit (Fig. 4A). In ethylene-treated fruit, the level of the 72 kDa protein was low right after treatment. Although it showed a slight increase at 2 DAT, the protein level decreased thereafter (Fig. 4B); the protein was barely detectable after the peak of endogenous ethylene production. In the case of the 68 kDa protein, the level was low after treatment. The protein level increased at 2 DAT and reached a maximum level at 4 DAT followed by a decrease at 6 and 8 DAT (Fig. 4B). Unlike the 72 kDa protein, the 68 kDa protein was maintained at a certain level after the peak of ethylene production (Fig. 4). On the other hand, 1-MCP treatment delayed both ethylene production and ripening (Fig. 5A). The peak of ethylene pro-

![Fig. 3. Endogenous ethylene production of control fruit during ripening. The fruit were ripened at 20°C. Five fruit were sampled on the indicated day and ethylene production was measured by gas chromatography. Vertical bars indicate the standard errors (n = 5).](image1)

![Fig. 4. Changes in endogenous ethylene production during ripening (A) and immunoblot analysis of α-L-arabinofuranosidase in ripening avocado fruit (B) treated by exogenous ethylene. The fruit were ripened at 20°C. Five fruit were sampled on the indicated day. Details of immunoblot and ethylene measurements are the same as in Figures 1 and 3, respectively. Vertical bars in Panel A indicate the standard errors (n = 5).](image2)

![Fig. 5. Changes in endogenous ethylene production during ripening (A) and immunoblot analysis of α-L-arabinofuranosidase in ripening avocado fruit (B) treated by 1-MCP. The fruit were ripened at 20°C. Five fruit were sampled on the indicated day. Details of immunoblot and ethylene measurements are the same as in Figures 1 and 3, respectively. Vertical bars in Panel A indicate the standard errors (n = 5).](image3)
duction shifted from 6 DAT to 9 DAT (Figs. 3 and 5A). There was an increase in the 72 kDa protein at 5 DAT. After reaching the peak at 7 DAT, it began to decrease at 9 DAT, when the peak of endogenous ethylene production was observed (Fig. 5B). In contrast, there was a notable increase in the 68 kDa protein at 9 DAT. Besides that time point, the protein level was constant through the experimental period (Fig. 5A, B).

These results described above indicate that ethylene production affects the accumulations of the two α-L-arabinofuranosidase proteins in opposite ways. Accumulation of the 72 kDa protein, which was detected before ethylene production, was negatively regulated by ethylene. Both exogenous and endogenous ethylene strongly suppressed its accumulation. In contrast, the 68 kDa protein showed a temporary accumulation in response to ethylene. The increase may be due to the elevation in the mRNA level; an up-regulation of α-L-arabinofuranosidase genes with ethylene action have been shown in peach and pear fruit (Hayama et al., 2006; Mwaniki et al., 2007). To date, only a cDNA clone encoding α-L-arabinofuranosidase has been isolated from avocado fruit (PaArf/Xyl3A) and the expression was observed in the fruit right after harvest when the ethylene production was still undetectable (Tateishi et al., 2015). At this point, it is uncertain whether the two proteins detected by immunoblot analysis were the products of PaArf/Xyl3A or not. However, our results unambiguously showed that both proteins were detected in the fruit right after harvest and the protein levels in the fruit were affected by ethylene action or a ripening signal.

**Effect of ethylene on total α-L-arabinofuranosidase activity**

In addition to the protein levels, we also examined the effect of exogenous ethylene or 1-MCP treatment on the α-L-arabinofuranosidase activity during avocado fruit ripening. α-L-Arabinofuranosidase activity was detected in the control fruit right after harvest (Fig. 6). The enzyme activity increased after endogenous ethylene production. The fluctuation in the activity was similar to our previous study (Tateishi et al., 2001). Exogenous ethylene treatment hastened the timing of increased enzyme activity; the increase in the activity was observed 2 days earlier than the control fruit. In contrast, 1-MCP treatment delayed the increase in the enzyme activity. In the 1-MCP treated fruit, there was only a slight change in the enzyme activity at 1 to 7 DAT. The enzyme activity increased after ethylene production initiated. Regardless of the treatment, an increase in the enzyme activity was observed concomitant with the timing of endogenous ethylene burst (Fig. 6). However, the increase in the activity was not consistent with the accumulation patterns of the two proteins detected. While the level of the 72 kDa protein reached a maximum before ethylene production, the highest accumulation of the 68 kDa protein was found at the peak of ethylene production (Figs. 1, 4–5). It is possible that the family 3 α-L-arabinofuranosidases gain higher activity upon ripening initiation. However, a more simple explanation would be the presence of other α-L-arabinofuranosidase protein(s), which do not cross-react with the family 3 Japanese pear antibody, in ripening avocado fruit. Given that the antibody cross-reacted at a taxonomic order between Rosales (Japanese pear) and Laurales (avocado), it is likely that no other family 3 α-L-arabinofuranosidases are present in the ripening avocado fruit. A portion of the increased enzyme activity during ripening could be derived from family 51 α-L-arabinofuranosidase rather than family 3 because expressions of family 51 α-L-arabinofuranosidase genes during fruit ripening have also been found in the fig (Ficus carica) (Owino et al., 2004) and strawberry (Rosli et al., 2009).

Loss of arabinosyl residues during fruit softening found in avocado (Brummell, 2006) should be caused by the action of α-L-arabinofuranosidase activity. GH family 3 α-L-arabinofuranosidases possess broad substrate specificities and the related gene expressions were detected not only in the fruit (Minic and Jouanin, 2006; Tateishi, 2008), indicating the pleiotropic functions of α-L-arabinofuranosidase on plant growth including fruit ripening. Also, family 51 α-L-arabinofuranosidase(s), which possess a different preference against natural substrates containing arabinosyl residue, may act on arabinose loss in ripening avocado fruit in coordination with those of family 3. We conclude that the two α-L-arabinofuranosidases examined in this study are subjected to opposite reg-
ulation under ethylene action or a ripening signal, indicating that they have specific functions in cell wall modification in ripening avocado fruit.

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