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Expression Analysis of *1-aminocyclopropane-1-carboxylic Acid Oxidase* Genes in Chitosan-Coated Banana

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ABSTRACT

Banana is a climacteric fruit in which ethylene plays an important role in the regulation of the ripening process. Though it is the most produced fruit in Indonesia, the current post-harvest technologies for exporting this fruit are not economically friendly. Chitosan is one of economical biopolymer for edible coating which can extend fruit shelf-life. However, little study focused on the effect of chitosan coating has been done on gene expression level. In this study, the expression levels of several *1-aminocyclopropan-1-carboxylic acid oxidase* (*ACO*) genes, which is an enzyme to convert 1-aminocyclopropan-1-carboxylic acid to ethylene in banana were analyzed on day 0, 1, 3, 5, 7, and 9 after ethylene treatment. As a result, one gene (ID: Ma01_t11540.1) had a similar expression pattern in both control and chitosan-coated bananas while the other genes (ID: Ma03_t02700.1, Ma05_t09360.1, Ma06_t02600.1, Ma10_t01130.1) showed different expression patterns. Among these genes, two genes (ID: Ma05_t09360.1, Ma10_t01130.1) were expressed higher than the other genes and the peak was observed on day 3. It was indicated that chitosan coating might activate the ethylene biosynthesis pathway in banana while it delayed fruit ripening.

1. Introduction

Post-harvest fruit is living organism and its commercial value decreases during the process of storage and transportation. Banana (*Musa acuminata*) is one of the most important crop plants consumed in many countries. It is a climacteric fruit and affected by respiration and ethylene production in post-harvest maturing process (Hubert and Didier 2012). The ethylene production of banana shows a sharp increase and decrease at the beginning of the climacteric period (Burg and Burg 1962; Karikari *et al.* 1979). This physiological climacteric characteristic ofbanana fruit leads to a fast ripening and a short shelf-life. Therefore, some post-harvest technologies had been developed to maintain banana fruit quality during storage and transportation and to extend its shelf-life. Existing well-known methods are controlled atmosphere (CA) Storage (Ahmad *et al.* 2011), modified atmosphere (MA) packaging and low temperature storage (Kudachikar *et al.* 2011) etc. However, these technologies require high cost and energy.

Edible coating is an alternative method that can extend the shelf life of fruit owing to its ability to prevent moisture loss, aroma loss, and inhibit the oxygen penetration to the plant tissue. In recent years, edible coating has received much attention because of its economical, simple, and biodegrad- able properties (Jianglian and Shaoying 2013). Chitosan is one of promising biopolymers for edible coating and has been categorized as Generally recognize as safe (GRAS) by Food and Drug Administration (FDA) (Jianglian and Shaoying 2013; Luo and Wang 2013). Chitosan is a polysaccharide obtained from deacetylation process of chitin, the second most abundant polysaccharide in nature after cellulose and can be found from many sources including exo-skeletons of crustaceans, insects, molluscs and fungi (Jianglian and Shaoying 2013; Luo and Wang 2013). It could reduce the respiration rate of fruit by forming coating on fruit and adjusting the permeability of carbon dioxide and oxygen (Jianglian and Shaoying 2013).

Though the effect of chitosan for fruit storage has been confirmed through chemical and physical analysis, a little study on its mechanism to delay fruit ripening has been conducted. Genetic approach could give an insight for the study on the mechanism of the delay of ripening by determining the molecular basis of gene action. It has been observed that particular genes are involved and play a role during ripening process, such as gene families associated

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with ethylene biosynthesis, cell wall degradation and so on (Asif *et al.* 2014). Ethylene is the dominant trigger for ripening in climacteric fruit including banana and affects the transcription and translation of many ripening-related genes (Alexander and Grierson 2002). Ethylene is naturally synthesized from ACC (1-aminocyclopropan-1-carboxylic acid) in fruits. The process is catalyzed by ACC oxidase (ACO) which is encoded by *ACO* genes (Kende 1993). The study of *ACO* gene expression could suggest if chitosan coating delays banana ripening by affecting ethylene biosynthesis or not.

The aim of this research is to study the mechanism of chitosan coating for delay of banana ripening by the analysis of *ACO* gene expression using real-time polymerase chain reaction (PCR). A good understanding of working genes involved in the ethylene biosynthesis is very important to reveal the mechanism of delaying fruit ripening and it would contribute to improve the existing post-harvest technology especially for storage of banana and also other fruits.

2. Materials and Methods

2.1. Sample Preparation and Observation

Hands of mature green Cavendish banana (*Musa acuminata AAA* group) were supplied by PT. Sewu Segar Nusantara, Indonesia. Hands were sectioned into fingers and were visually selected for uniformity in size, color, and absence of physical damage and fungal infection. Chitosan food grade (High molecular weight and 85-89% deacetylated) was purchased from Biotech Surindo, Indonesia. All chemicals used in the experiment were analytical grade.

2.2. Preparation of Chitosan Coating

1.25% chitosan was used as an edible coating of banana. 1.25% (w/v) chitosan solution was prepared by dissolving the corresponding amount of chitosan in the solution containing 1% (w/v) acetic acid. The solution was agitated using stirrer until being homogeneous. The pH of solution was adjusted to 5.5 with 1 M NaOH. The coating was done by infusing hands to the chitosan solution for 2 minutes, wind-drying at room temperature. Then they were stored in a storage area at 25^{0}C. Observation was done on days 0, 1, 3, 5, 7, 9, 11. The changes of peel color were documented by taking photos for each day of observation.

2.3. Conversion of Starch Into Sugar

Conversion of starch into sugar was assessed by using the starch iodine test. The mid-point of banana was cut transversely about 2-3 cm thick and then peel was separated from the pulp. The cut surface of the banana was dipped at a depth of 5 mm for 5 seconds in starch-iodine staining solution. The starch pattern of each fruit was assessed by comparing the stained cut surface with starch iodine staining chart for bananas which developed by Blankenship *et al.* 1993. Starch-iodine staining solution was prepared by dissolving 1% potassium iodide (dissolve first in small amount of hot water) and 0.25% iodine in distilled water.

2.4. Pulp to Peel Ratio

Pulp to peel ratio was measured by calculating pulp weight divided by peel weight. Pulp and peel were separated and weighed individually as described (Karmawan *et al.* 2009; Dwivany *et al.* 2016).

2.5. Total Soluble Solids (TSS)

The TSS content of banana fruit was determined by using a refractometer (Atago) and performed as described (Pratiwi *et al.* 2015). Results were expressed as degree Brix (°Brix). Briefly, 15 g banana fruit pulp in each treatment was homogenized using a blender with 45 mL of distilled water. The mixture was centrifuged at 14000 rpm for 5 min. A few drops of the filtrate were then placed on the prism of the refractometer before reading. The refractometer was calibrated with distilled water to give a 0ºBrix reading at each measurement.

2.6. RNA Total Extraction and Library Preparation for qPCR

RNA isolation was done for samples of day 0, 1, 3, 5, 7, and 9. Total RNA was extracted from ground banana using the extraction EB buffer (2% CTAB; 2% PVP; 100 mM Tris-HCl pH 8; 25 mM EDTA; 2 M NaCl; 2% 2-mercaptoethanol) and recovered using lithium chloride 2M as described by Cordeiro *et al.* 2008. The derived RNA was confirmed by electrophoresis and its concentration was confirmed by using Nanodrop. RNase-free DNase I (Thermo Fisher Scientific) was added and cDNA was synthesized using iScript™ cDNA Synthesis kit (BIORAD).

2.7. Quantitative Real-Time PCR (qPCR)

Synthesized cDNA were firstly amplified by polymerase chain reaction (PCR) before qPCR using gene-specific primers for each *ACO* genes shown in Table 1. Sequences used for designing primers were referred to Banana Genome Hub (http://banana-genome-hub.southgreen.fr/home1). Primers were designed by using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and confirmed by using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).PCR was performed on the ABI Veriti® thermocycler with GoTaq® Green Master Mix (Promega). The cycle of PCR is as shown in Figure 1.

Table 1. Primers of *ACO* genes for qPCR

Gene ID* (Genbank ID)	Location*	Coding protein*	primerF	primerR
Ma01_t11540.1 (XM_009414995.1)	chr01:8344228-8345611	ACO1-like	CACGAACGGCAAGTACAAGA	CTGTTACCGTGGCCTTCATT
Ma03_t02700.1 (XM_009392868.1)	chr03:1835668-1839116	ACO-like	CGGTCATCGATTTCTCCAAG	TCGGAGCTGACCTTCTTCAC
Ma05_t09360.1 (XM_009402256.1)	chr05:6781198-6784233	ACO1-like	TCGACTGGGAGACCACCTAC	GAGGCGCAAATGTCTTCTTC
Ma06_t02600.1 (XM_009404647.1)	chr06:2004016-2005240	ACO1-like	GGAGTGATGGAGGAGGTGAA	GATGGCGGTAGAAGAAGCTG
Ma10_t01130.1 (XM_009422017.1)	chr10:4027373-4031606	ACO homolog 3-like	GAGGATGGGGAGTCTGAGTG	TCCAGGGTTGAAGAAGGTTG

*Banana Genome Hub (http://banana-genome-hub.southgreen.fr/home1)

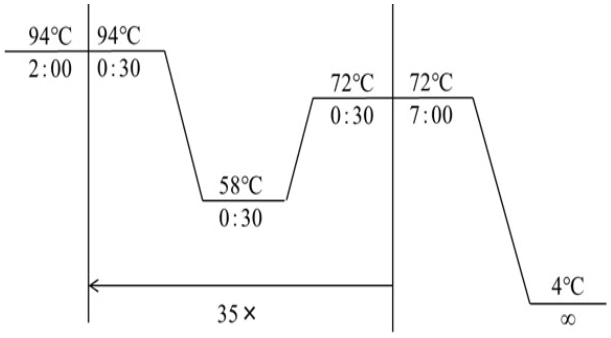

Figure 1. Cycle of PCR

The gene expression analysis was performed by real-time PCR on thermocycler Bio-Rad iCycler® CFX 96 TM connected with iQ™5 Real-Time PCR Detection Systems using SsoFast™ EvaGreen® Supermix. In each qPCR analysis, three samples were used for triplicate. qPCR cycle condition was 95°C for 3 minutes and 40 cycles of 95°C for 30 seconds and 58°C for 30 seconds, followed by a melting curve from 58°C to 95°C (increment 0.5°C for 5 minutes).

Relative expression levels were normalized by the internal control gene, *MaGADPH* (glyceraldehyde-3-phosphate-dehydrogenase). Primers for control genes were MaGADPH_F 5'-TCAACGACCCCTTCATCAC-3' and MaGADPH_R 5'-AGCAGCCTTGTCCTTGTCA-3' (Handayani and Dwivany, 2012). The relative expression level was calculated using obtained Cq value (quantification cycle) by $2^{-\Delta\Delta Ct}$ method as mentioned (Livak and Schmittgen 2001).

3. Results

3.1. The Effect of Chitosan Coating on the Physicochemical Characteristics of Banana

The effect of chitosan coating on the physicochemical characteristics of banana is shown in Figure 2. As observed in Figure 2a, uncoated banana (control) and chitosan-coated banana had different ripening speed based on its peel color. On day 3, uncoated banana was on stage 5 (yellow with green tips), while coated banana was on stage 2 (light green). Banana treated with chitosan showed a slower deterioration compared to uncoated banana. On day 9, uncoated banana already started to decay while banana treated with chitosan was still on stage 4 (more yellow than green). The effect of chitosan coating on starch content of banana fruit is shown in Figure 2b. In uncoated banana, starch content of banana fruit started to decrease on day 5. Chitosan-coated banana started to convert starch to sugar on day 7. In this study, there was not a significant difference in pulp to peel ratio between uncoated banana and chitosan-coated banana (Figure 2c). The effect of chitosan coating on total soluble solid (TSS) value is shown in Figure 2d. TSS value of uncoated banana increased rapidly until day 3 and gradually increased from day 3 to day 11. As shown in the figure, TSS value of coated banana was always lower than that of uncoated banana.

3.2. The Effect of Chitosan Coating on the Expression Level of ACO Genes

The expression level of *1-aminocyclopropan-1-carboxylic acid oxidase* (*ACO*) gene was analyzed. ACO is an enzyme that converts 1-aminocyclopropan-1-carboxylic acid (ACC) to ethylene that is the dominant trigger for ripening in climacteric fruit including banana (Figure 3).

The expression levels of 5 *ACO* genes (shown in Table 1) were analyzed using real-time PCR. The expressions for each gene were examined on day 0, 1, 3, 5, 7, and 9. Various expression patterns were observed among these genes. The mRNA expression levels of each ACO gene are shown in Figure 4. In uncoated banana, the expression levels of 3 genes (Ma01_t11540.1, Ma05_t09360.1, Ma06_t02600.1) showed the similar pattern decreasing from day 0 during ripening. The other 2 genes (Ma03_t02700.1, Ma10_t01130.1) had the similar expression pattern. The expression level of those genes had a peak at day 5. When compared uncoated and chitosan-

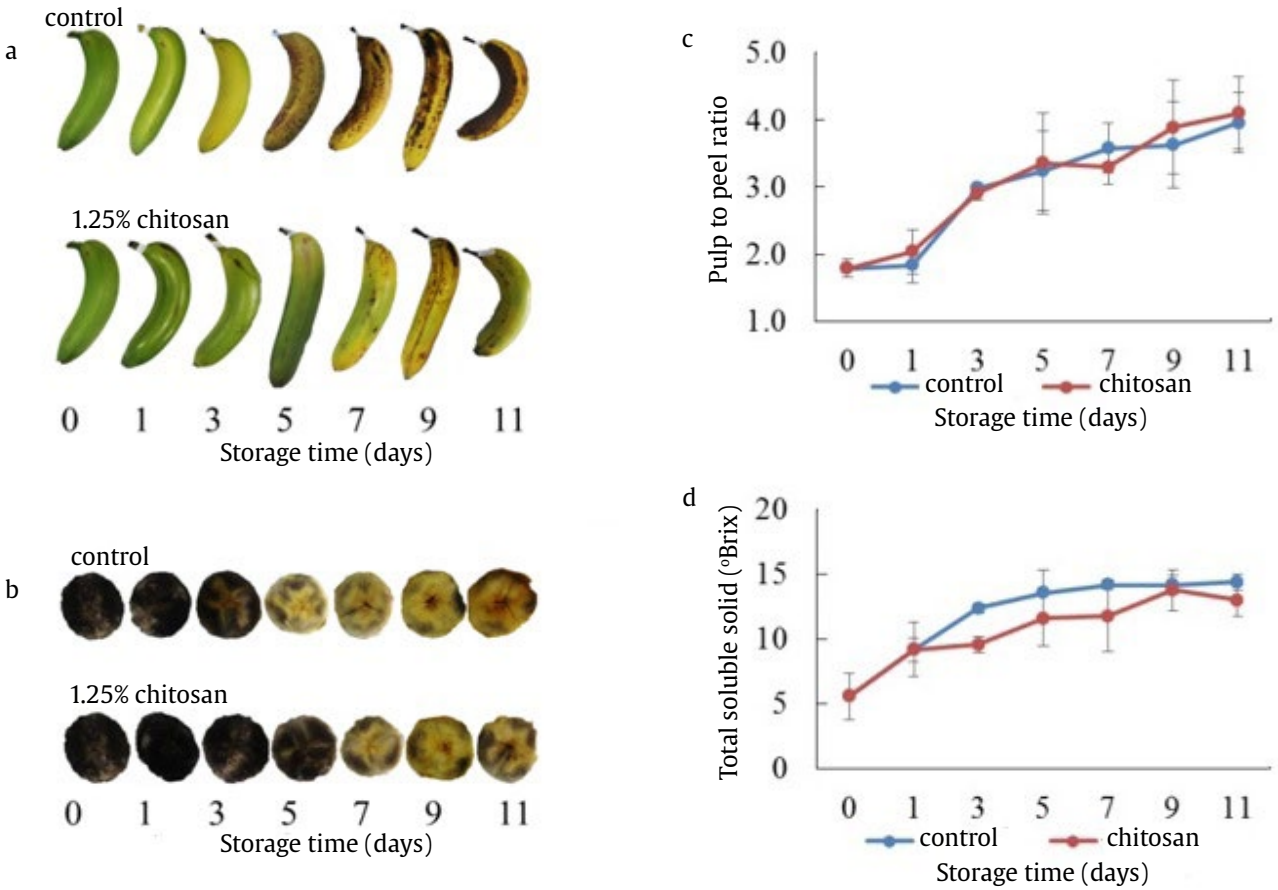

Figure 2. Physicochemical characteristics of uncoated and coated bananas with 1.25% chitosan solution during ripening from day 0 to 11. (a) Color changes of peel (b) Starch conversion pattern into sugars confirmed by using iodine reaction (c) Pulp to peel ratio based on weight. Blue: uncoated, Red: chitosan-coated samples. Error bars indicate standard deviation (SD). n = 3 (d) Total soluble solid measured by refractometer. Results were expressed as degree Brix (°Brix). Blue: uncoated, Red: chitosan-coated samples. Error bars indicate SD. n = 3

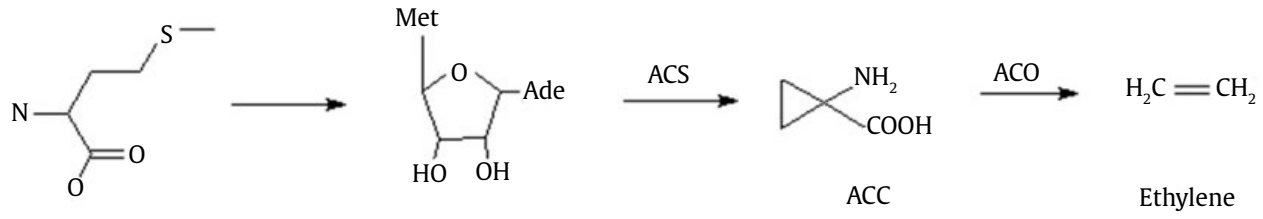

Figure 3. The ethylene biosynthesis pathway in plants in general. Ethylene is produced from methionine, which is first converted to S-adenosyl methionine (AdoMet). The next two steps are the conversion of AdoMet to ACC by ACS and the oxidative cleavage of ACC by ACO to form ethylene. (Met: Methionine, ACC: 1-Aminocyclopropane-1-carboxylic acid, ACS: ACC synthase, ACO: ACC oxidase) (Adapted from Song and Liu 2015)

coated group, one gene (Ma01_t11540.1) had a similar expression pattern in both uncoated and chitosan-coated condition. The other genes (Ma03_t02700.1, Ma05_t09360.1, Ma06_t02600.1, Ma10_t01130.1) showed different expression patterns between uncoated and chitosan-coated groups. In chitosan-coated banana, the expression level of 2 genes (Ma03_t02700.1, Ma06_t02600.1) decreased from day 0 and increased twice, at day 3 and day 9. The other 2 genes (Ma05_t09360.1, Ma10_t01130.1) had a peak at day 3 and their expression levels were higher than other genes (Figure 5). These 2 genes were highly expressed in chitosan-coated banana compared to uncoated banana and were considered as the most interesting genes.

4. Discussion

Chitosan coating could provide a good effect on postharvest quality of banana including color change of peel, starch content, TSS as reported previously. Color change of banana peel occurs due to degradation

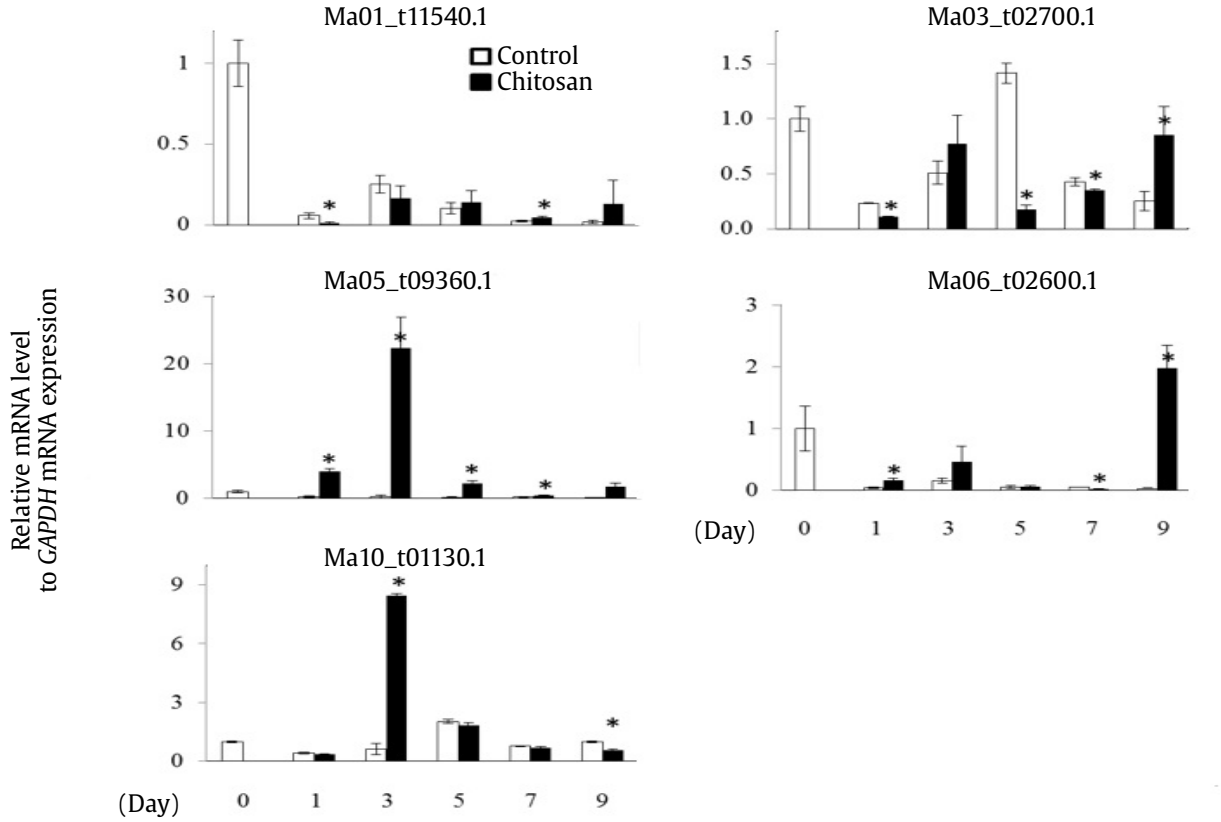

Figure 4. mRNA expression level of each ACO gene. Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from both uncoated and chitosan-coated banana fruit tissue during ripening on day 0, 1, 3, 5, 7 and 9. Each relative transcript abundance was normalized by MaGADPH using $2^{-\Delta\Delta Ct}$ method. *; $P < 0.05$ versus uncoated group. Two-tailed t-tests were conducted for each day pair of uncoated and chitosan-coated samples. Error bars indicate SD. n = 3

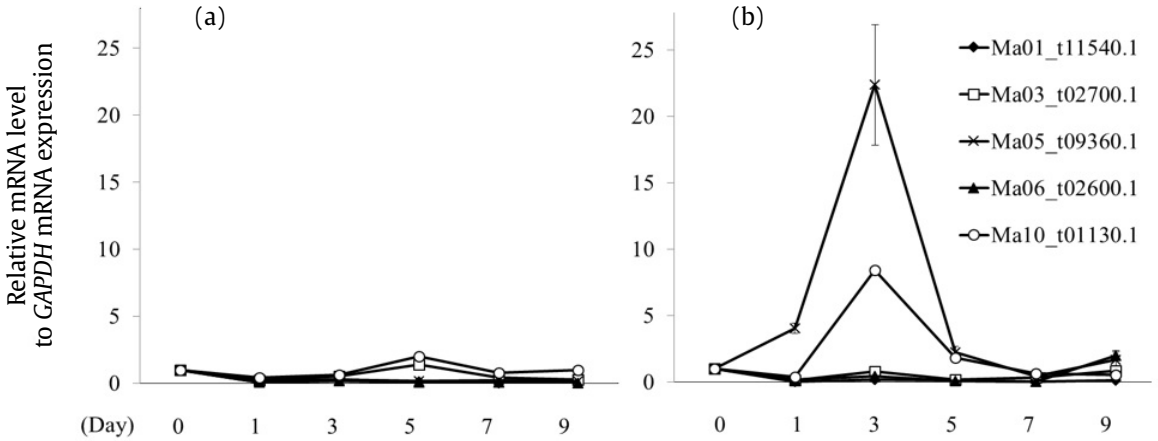

Figure 5. mRNA expression level of each *ACO* gene in uncoated banana (a) and chitosan-coated banana (b). Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from fruit tissue during ripening on day 0, 1, 3, 5, 7, and 9. Each relative transcript abundance was normalized by *MaGADPH* using $2^{-\Delta\Delta Ct}$ method. Error bars indicate SD. n = 3

of chlorophyll during ripening and it was delayed by chitosan treatment. Pulp to peel ratio is commonly used to measure the degree of ripening and it was reported that the pulp to peel ratio of banana coated with chitosan was lower than uncoated banana (Pratiwi *et al.* 2015). TSS value increases during ripening because most of the soluble solids in banana is sugar and it's converted from starch in the ripening process (Dadzie and Orchard 1997). It was also reported that TSS value of chitosan-coated banana increased slowly (Maqbool *et al.* 2011). In this study, chitosan coating delayed color change and reduced the increase of TSS value. This result indicated that chitosan coating can extend the shelf-life of banana. It has been reported that chitosan coating can extend the shelf-life of fruits. Therefore, fruit coating with chitosan had also been reported by many researchers such as strawberry

(Eshghi *et al.* 2014), lychee (Dong *et al.* 2004), longan (Jiang and Li 2000), mango (Chien *et al.* 2007). It has been confirmed that chitosan coating retarded ripening and reduced the respiration rate in longan fruit (Jiang and Li 2000). Chitosan coating may have a role to reduce the gas exchange surrounded the fruit. The low level of O_2 and high level of CO_2 can inhibit degradation of chlorophyll in banana peel (Sorrentino *et al.* 2007). The conversion of starch into sugar is also affected by level of O_2 and CO_2. Low level of O_2 and high level of CO_2 can inhibit the activities of enzymes involved in hydrolysis of starch (Maqbool *et al.* 2011). Furthermore, that O_2 and CO_2 condition can inhibit the production of ethylene, a trigger of banana ripening (Sorrentino *et al.* 2007).

According to the real-time PCR results for *ACO* genes, except one gene (Ma01_t11540.1), there was a difference between the expression pattern of uncoated and chitosan-coated banana. Interestingly, high expression level of two genes (Ma05_t09360.1, Ma10_t01130.1) was observed in chitosan-coated banana and that showed ethylene synthesis pathway might be activated in coated banana. It was suggested that some of *ACO* genes were affected by chitosan coating and as a consequence, ethylene biosynthesis pathway was activated. However, the reduction of ethylene production has been reported as a result of coating with edible films for banana (Banks 1984) and other fruits, such as tomato fruit (Nisperos and Baldwin 1988). Therefore, it was considered that transcription of *ACO* genes was activated but the downstream process, the ethylene production, might be inhibited. Since chitosan coating inhibits O_2 penetration (Jianglian and Shaoying 2013) and ACO needs O_2 as a substrate when it converts ACC to ethylene (Kende 1993), it was suggested that chitosan coating might inhibit the ethylene production by adjusting the permeability of surrounded O_2.

This study gave a new insight about gene expression that is related to ethylene biosynthesis pathway. Though chitosan coating delayed banana ripening, high expression of two *ACO* genes (Ma05_t09360.1, Ma10_t01130.1) suggested the activation of ethylene biosynthesis pathway. Further expression analysis on both upstream and downstream genes in ethylene biosynthesis is required to reveal the mechanism clearly.

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with ethylene biosynthesis, cell wall degradation and so on (Asif *et al.* 2014). Ethylene is the dominant trigger for ripening in climacteric fruit including banana and affects the transcription and translation of many ripening-related genes (Alexander and Grierson 2002). Ethylene is naturally synthesized from ACC (1-aminocyclopropan-1-carboxylic acid) in fruits. The process is catalyzed by ACC oxidase (ACO) which is encoded by *ACO* genes (Kende 1993). The study of *ACO* gene expression could suggest if chitosan coating delays banana ripening by affecting ethylene biosynthesis or not.

The aim of this research is to study the mechanism of chitosan coating for delay of banana ripening by the analysis of *ACO* gene expression using real-time polymerase chain reaction (PCR). A good understanding of working genes involved in the ethylene biosynthesis is very important to reveal the mechanism of delaying fruit ripening and it would contribute to improve the existing post-harvest technology especially for storage of banana and also other fruits.

2. Materials and Methods

2.1. Sample Preparation and Observation

Hands of mature green Cavendish banana (*Musa acuminata AAA* group) were supplied by PT. Sewu Segar Nusantara, Indonesia. Hands were sectioned into fingers and were visually selected for uniformity in size, color, and absence of physical damage and fungal infection. Chitosan food grade (High molecular weight and 85-89% deacetylated) was purchased from Biotech Surindo, Indonesia. All chemicals used in the experiment were analytical grade.

2.2. Preparation of Chitosan Coating

1.25% chitosan was used as an edible coating of banana. 1.25% (w/v) chitosan solution was prepared by dissolving the corresponding amount of chitosan in the solution containing 1% (w/v) acetic acid. The solution was agitated using stirrer until being homogeneous. The pH of solution was adjusted to 5.5 with 1 M NaOH. The coating was done by infusing hands to the chitosan solution for 2 minutes, wind-drying at room temperature. Then they were stored in a storage area at 25^{0}C. Observation was done on days 0, 1, 3, 5, 7, 9, 11. The changes of peel color were documented by taking photos for each day of observation.

2.3. Conversion of Starch Into Sugar

Conversion of starch into sugar was assessed by using the starch iodine test. The mid-point of banana was cut transversely about 2-3 cm thick and then peel was separated from the pulp. The cut surface of the banana was dipped at a depth of 5 mm for 5 seconds in starch-iodine staining solution. The starch pattern of each fruit was assessed by comparing the stained cut surface with starch iodine staining chart for bananas which developed by Blankenship *et al.* 1993. Starch-iodine staining solution was prepared by dissolving 1% potassium iodide (dissolve first in small amount of hot water) and 0.25% iodine in distilled water.

2.4. Pulp to Peel Ratio

Pulp to peel ratio was measured by calculating pulp weight divided by peel weight. Pulp and peel were separated and weighed individually as described (Karmawan *et al.* 2009; Dwivany *et al.* 2016).

2.5. Total Soluble Solids (TSS)

The TSS content of banana fruit was determined by using a refractometer (Atago) and performed as described (Pratiwi *et al.* 2015). Results were expressed as degree Brix (°Brix). Briefly, 15 g banana fruit pulp in each treatment was homogenized using a blender with 45 mL of distilled water. The mixture was centrifuged at 14000 rpm for 5 min. A few drops of the filtrate were then placed on the prism of the refractometer before reading. The refractometer was calibrated with distilled water to give a 0ºBrix reading at each measurement.

2.6. RNA Total Extraction and Library Preparation for qPCR

RNA isolation was done for samples of day 0, 1, 3, 5, 7, and 9. Total RNA was extracted from ground banana using the extraction EB buffer (2% CTAB; 2% PVP; 100 mM Tris-HCl pH 8; 25 mM EDTA; 2 M NaCl; 2% 2-mercaptoethanol) and recovered using lithium chloride 2M as described by Cordeiro *et al.* 2008. The derived RNA was confirmed by electrophoresis and its concentration was confirmed by using Nanodrop. RNase-free DNase I (Thermo Fisher Scientific) was added and cDNA was synthesized using iScript™ cDNA Synthesis kit (BIORAD).

2.7. Quantitative Real-Time PCR (qPCR)

Synthesized cDNA were firstly amplified by polymerase chain reaction (PCR) before qPCR using gene-specific primers for each *ACO* genes shown in Table 1. Sequences used for designing primers were referred to Banana Genome Hub (http://banana-genome-hub.southgreen.fr/home1). Primers were designed by using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and confirmed by using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).PCR was performed on the ABI Veriti® thermocycler with GoTaq® Green Master Mix (Promega). The cycle of PCR is as shown in Figure 1.

Table 1. Primers of *ACO* genes for qPCR

Gene ID* (Genbank ID)	Location*	Coding protein*	primerF	primerR
Ma01_t11540.1 (XM_009414995.1)	chr01:8344228-8345611	ACO1-like	CACGAACGGCAAGTACAAGA	CTGTTACCGTGGCCTTCATT
Ma03_t02700.1 (XM_009392868.1)	chr03:1835668-1839116	ACO-like	CGGTCATCGATTTCTCCAAG	TCGGAGCTGACCTTCTTCAC
Ma05_t09360.1 (XM_009402256.1)	chr05:6781198-6784233	ACO1-like	TCGACTGGGAGACCACCTAC	GAGGCGCAAATGTCTTCTTC
Ma06_t02600.1 (XM_009404647.1)	chr06:2004016-2005240	ACO1-like	GGAGTGATGGAGGAGGTGAA	GATGGCGGTAGAAGAAGCTG
Ma10_t01130.1 (XM_009422017.1)	chr10:4027373-4031606	ACO homolog 3-like	GAGGATGGGGAGTCTGAGTG	TCCAGGGTTGAAGAAGGTTG

*Banana Genome Hub (http://banana-genome-hub.southgreen.fr/home1)

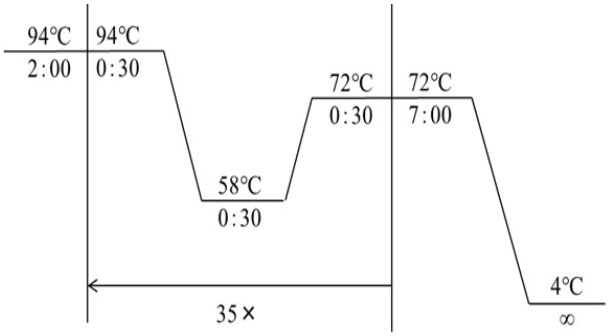

Figure 1. Cycle of PCR

The gene expression analysis was performed by real-time PCR on thermocycler Bio-Rad iCycler® CFX 96 TM connected with iQ™5 Real-Time PCR Detection Systems using SsoFast™ EvaGreen® Supermix. In each qPCR analysis, three samples were used for triplicate. qPCR cycle condition was 95°C for 3 minutes and 40 cycles of 95°C for 30 seconds and 58°C for 30 seconds, followed by a melting curve from 58°C to 95°C (increment 0.5°C for 5 minutes).

Relative expression levels were normalized by the internal control gene, *MaGADPH* (glyceraldehyde-3-phosphate-dehydrogenase). Primers for control genes were MaGADPH_F 5'-TCAACGACCCCTTCATCAC-3' and MaGADPH_R 5'-AGCAGCCTTGTCCTTGTCA-3' (Handayani and Dwivany, 2012). The relative expression level was calculated using obtained Cq value (quantification cycle) by $2^{-\Delta\Delta Ct}$ method as mentioned (Livak and Schmittgen 2001).

3. Results

3.1. The Effect of Chitosan Coating on the Physicochemical Characteristics of Banana

The effect of chitosan coating on the physicochemical characteristics of banana is shown in Figure 2. As observed in Figure 2a, uncoated banana (control) and chitosan-coated banana had different ripening speed based on its peel color. On day 3, uncoated banana was on stage 5 (yellow with green tips), while coated banana was on stage 2 (light green). Banana treated with chitosan showed a slower deterioration compared to uncoated banana. On day 9, uncoated banana already started to decay while banana treated with chitosan was still on stage 4 (more yellow than green). The effect of chitosan coating on starch content of banana fruit is shown in Figure 2b. In uncoated banana, starch content of banana fruit started to decrease on day 5. Chitosan-coated banana started to convert starch to sugar on day 7. In this study, there was not a significant difference in pulp to peel ratio between uncoated banana and chitosan-coated banana (Figure 2c). The effect of chitosan coating on total soluble solid (TSS) value is shown in Figure 2d. TSS value of uncoated banana increased rapidly until day 3 and gradually increased from day 3 to day 11. As shown in the figure, TSS value of coated banana was always lower than that of uncoated banana.

3.2. The Effect of Chitosan Coating on the Expression Level of ACO Genes

The expression level of *1-aminocyclopropan-1-carboxylic acid oxidase* (*ACO*) gene was analyzed. ACO is an enzyme that converts 1-aminocyclopropan-1-carboxylic acid (ACC) to ethylene that is the dominant trigger for ripening in climacteric fruit including banana (Figure 3).

The expression levels of 5 *ACO* genes (shown in Table 1) were analyzed using real-time PCR. The expressions for each gene were examined on day 0, 1, 3, 5, 7, and 9. Various expression patterns were observed among these genes. The mRNA expression levels of each ACO gene are shown in Figure 4. In uncoated banana, the expression levels of 3 genes (Ma01_t11540.1, Ma05_t09360.1, Ma06_t02600.1) showed the similar pattern decreasing from day 0 during ripening. The other 2 genes (Ma03_t02700.1, Ma10_t01130.1) had the similar expression pattern. The expression level of those genes had a peak at

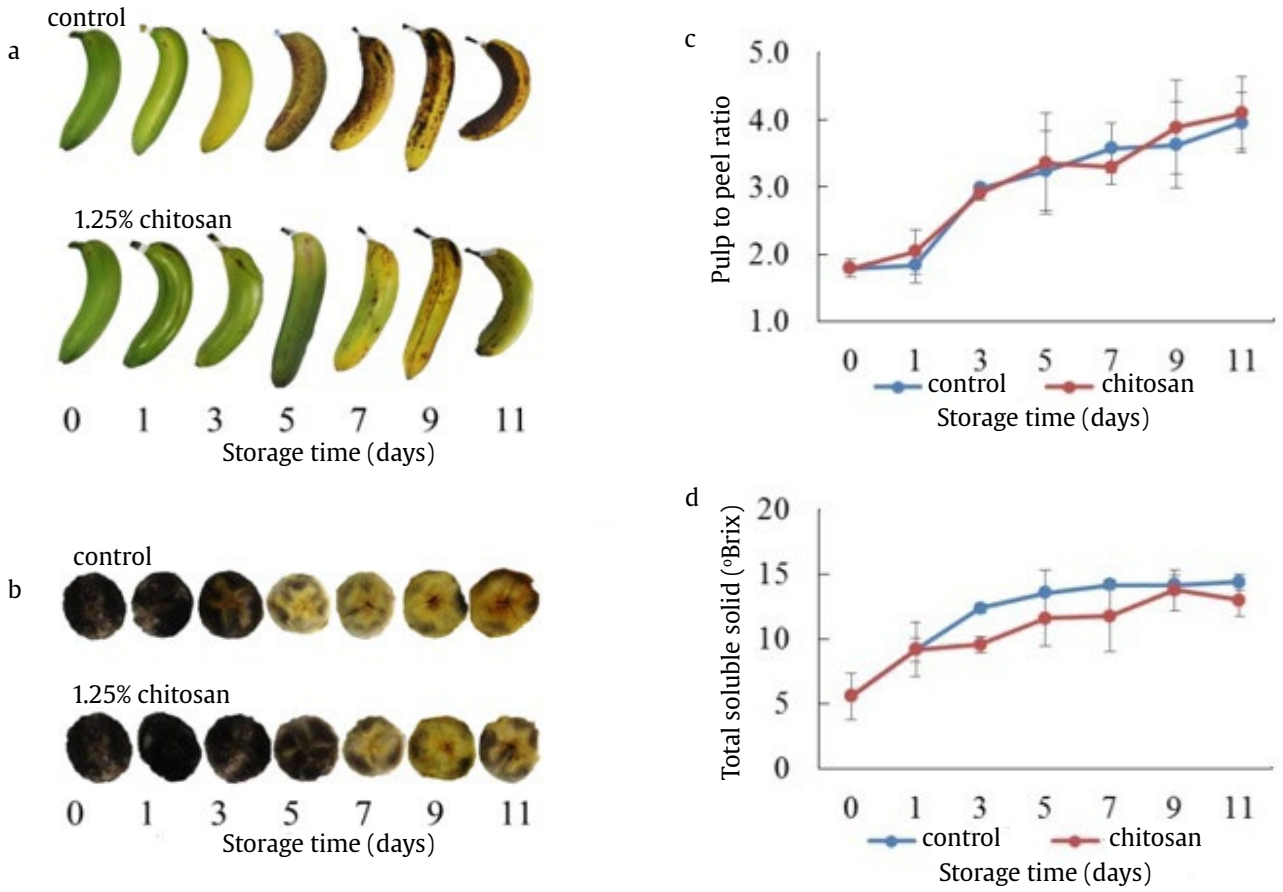

Figure 2. Physicochemical characteristics of uncoated and coated bananas with 1.25% chitosan solution during ripening from day 0 to 11. (a) Color changes of peel (b) Starch conversion pattern into sugars confirmed by using iodine reaction (c) Pulp to peel ratio based on weight. Blue: uncoated, Red: chitosan-coated samples. Error bars indicate standard deviation (SD). n = 3 (d) Total soluble solid measured by refractometer. Results were expressed as degree Brix (°Brix). Blue: uncoated, Red: chitosan-coated samples. Error bars indicate SD. n = 3

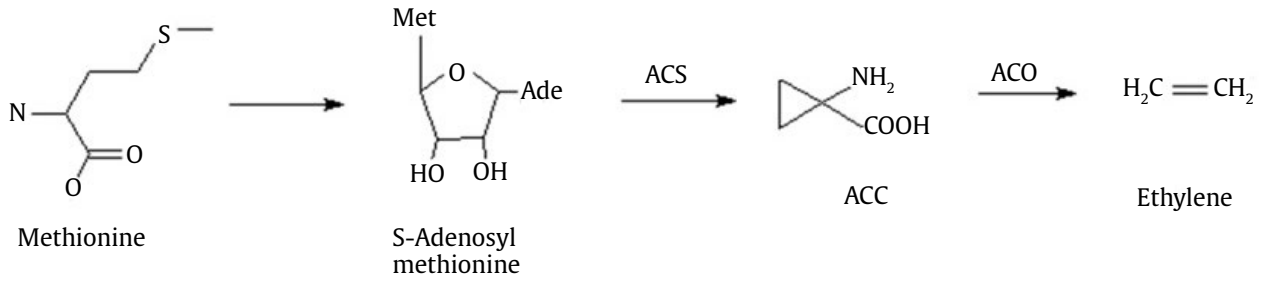

Figure 3. The ethylene biosynthesis pathway in plants in general. Ethylene is produced from methionine, which is first converted to S-adenosyl methionine (AdoMet). The next two steps are the conversion of AdoMet to ACC by ACS and the oxidative cleavage of ACC by ACO to form ethylene. (Met: Methionine, ACC: 1-Aminocyclopropane-1-carboxylic acid, ACS: ACC synthase, ACO: ACC oxidase) (Adapted from Song and Liu 2015)

day 5. When compared uncoated and chitosan-coated group, one gene (Ma01_t11540.1) had a similar expression pattern in both uncoated and chitosan-coated condition. The other genes (Ma03_t02700.1, Ma05_t09360.1, Ma06_t02600.1, Ma10_t01130.1) showed different expression patterns between uncoated and chitosan-coated groups. In chitosan-coated banana, the expression level of 2 genes (Ma03_t02700.1, Ma06_t02600.1) decreased from day 0 and increased twice, at day 3 and day 9. The other 2 genes (Ma05_t09360.1, Ma10_t01130.1) had a peak at day 3 and their expression levels were higher than other genes (Figure 5). These 2 genes were highly expressed in chitosan-coated banana compared to uncoated banana and were considered as the most interesting genes.

4. Discussion

Chitosan coating could provide a good effect on postharvest quality of banana including color change of peel, starch content, TSS as reported

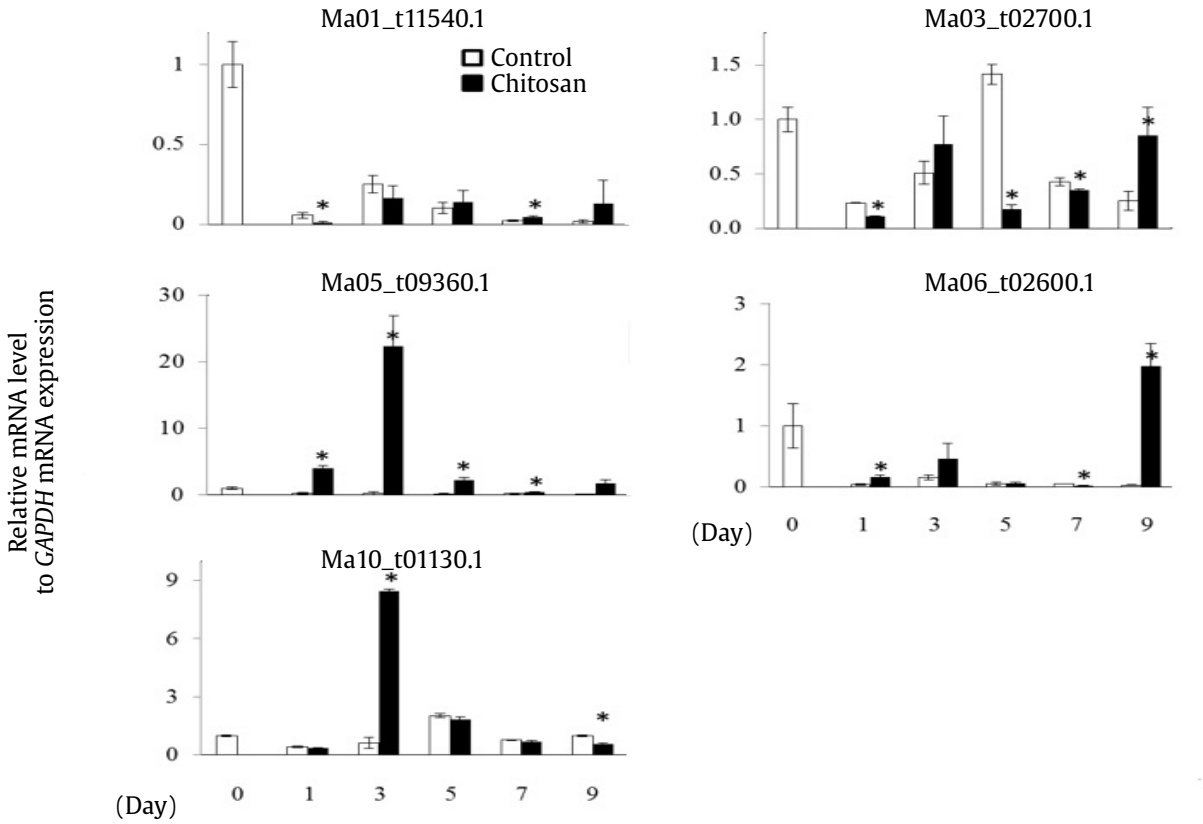

Figure 4. mRNA expression level of each ACO gene. Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from both uncoated and chitosan-coated banana fruit tissue during ripening on day 0, 1, 3, 5, 7 and 9. Each relative transcript abundance was normalized by MaGADPH using $2^{-\Delta\Delta Ct}$ method. *; $P < 0.05$ versus uncoated group. Two-tailed t-tests were conducted for each day pair of uncoated and chitosan-coated samples. Error bars indicate SD. n = 3

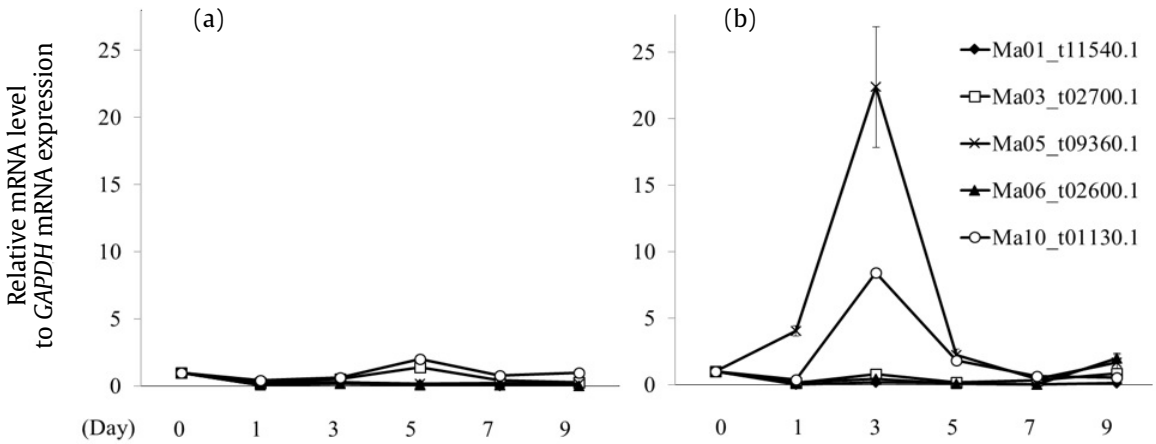

Figure 5. mRNA expression level of each *ACO* gene in uncoated banana (a) and chitosan-coated banana (b). Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from fruit tissue during ripening on day 0, 1, 3, 5, 7, and 9. Each relative transcript abundance was normalized by *MaGADPH* using $2^{-\Delta\Delta Ct}$ method. Error bars indicate SD. n = 3

previously. Color change of banana peel occurs due to degradation of chlorophyll during ripening and it was delayed by chitosan treatment. Pulp to peel ratio is commonly used to measure the degree of ripening and it was reported that the pulp to peel ratio of banana coated with chitosan was lower than uncoated banana (Pratiwi *et al.* 2015). TSS value increases during ripening because most of the soluble solids in banana is sugar and it's converted from starch in the ripening process (Dadzie and Orchard 1997). It was also reported that TSS value of chitosan-coated banana increased slowly (Maqbool *et al.* 2011). In this study, chitosan coating delayed color change and reduced the increase of TSS value. This result indicated that chitosan coating can extend the shelf-life of banana. It has been reported that chitosan coating can extend the shelf-life of fruits. Therefore, fruit coating with chitosan had also been

reported by many researchers such as strawberry (Eshghi *et al.* 2014), lychee (Dong *et al.* 2004), longan (Jiang and Li 2000), mango (Chien *et al.* 2007). It has been confirmed that chitosan coating retarded ripening and reduced the respiration rate in longan fruit (Jiang and Li 2000). Chitosan coating may have a role to reduce the gas exchange surrounded the fruit. The low level of O_2 and high level of CO_2 can inhibit degradation of chlorophyll in banana peel (Sorrentino *et al.* 2007). The conversion of starch into sugar is also affected by level of O_2 and CO_2. Low level of O_2 and high level of CO_2 can inhibit the activities of enzymes involved in hydrolysis of starch (Maqbool *et al.* 2011). Furthermore, that O_2 and CO_2 condition can inhibit the production of ethylene, a trigger of banana ripening (Sorrentino *et al.* 2007).

According to the real-time PCR results for *ACO* genes, except one gene (Ma01_t11540.1), there was a difference between the expression pattern of uncoated and chitosan-coated banana. Interestingly, high expression level of two genes (Ma05_t09360.1, Ma10_t01130.1) was observed in chitosan-coated banana and that showed ethylene synthesis pathway might be activated in coated banana. It was suggested that some of *ACO* genes were affected by chitosan coating and as a consequence, ethylene biosynthesis pathway was activated. However, the reduction of ethylene production has been reported as a result of coating with edible films for banana (Banks 1984) and other fruits, such as tomato fruit (Nisperos and Baldwin 1988). Therefore, it was considered that transcription of *ACO* genes was activated but the downstream process, the ethylene production, might be inhibited. Since chitosan coating inhibits O_2 penetration (Jianglian and Shaoying 2013) and ACO needs O_2 as a substrate when it converts ACC to ethylene (Kende 1993), it was suggested that chitosan coating might inhibit the ethylene production by adjusting the permeability of surrounded O_2.

This study gave a new insight about gene expression that is related to ethylene biosynthesis pathway. Though chitosan coating delayed banana ripening, high expression of two *ACO* genes (Ma05_t09360.1, Ma10_t01130.1) suggested the activation of ethylene biosynthesis pathway. Further expression analysis on both upstream and downstream genes in ethylene biosynthesis is required to reveal the mechanism clearly.

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