

## 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 of banana 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 biodegradable 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°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 0°Brix 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	CACGAACGGCAAGT-CAAGA	CTGTTACCGTGGCCT-TCATT
Ma03_t02700.1 (XM_009392868.1)	chr03:1835668-1839116	ACO-like	CGGTATCGATTCTC-CAAG	TGGGAGCTGACCTTCT-TCAC
Ma05_t09360.1 (XM_009402256.1)	chr05:6781198-6784233	ACO1-like	TCGACTGGGAGAC-CACCTAC	GAGGCGCAAATGTCT-TCTTC
Ma06_t02600.1 (XM_009404647.1)	chr06:2004016-2005240	ACO1-like	GGAGTGATGGAG-GAGGTGAA	GATGGCGGTAGAA-GAAGCTG
Ma10_t01130.1 (XM_009422017.1)	chr10:4027373-4031606	ACO homolog 3-like	GAGGATGGGGAGTCT-GAGTG	TCCAGGGTTGAAGAA-GGTTG

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

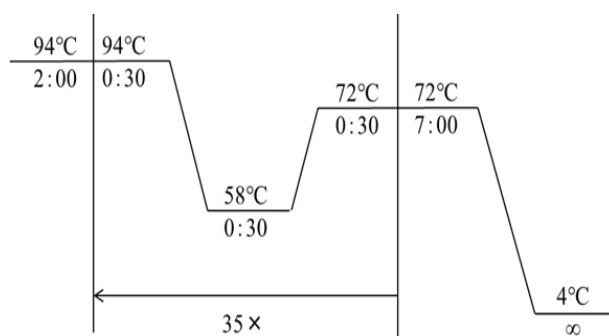


Figure 1. Cycle of PCR

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'-AGCAGCCTTGCTTGTCA-3' (Handayani and Dwivany, 2012). The relative expression level was calculated using obtained Cq value (quantification cycle) by  $2^{-\Delta\Delta Cq}$  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-

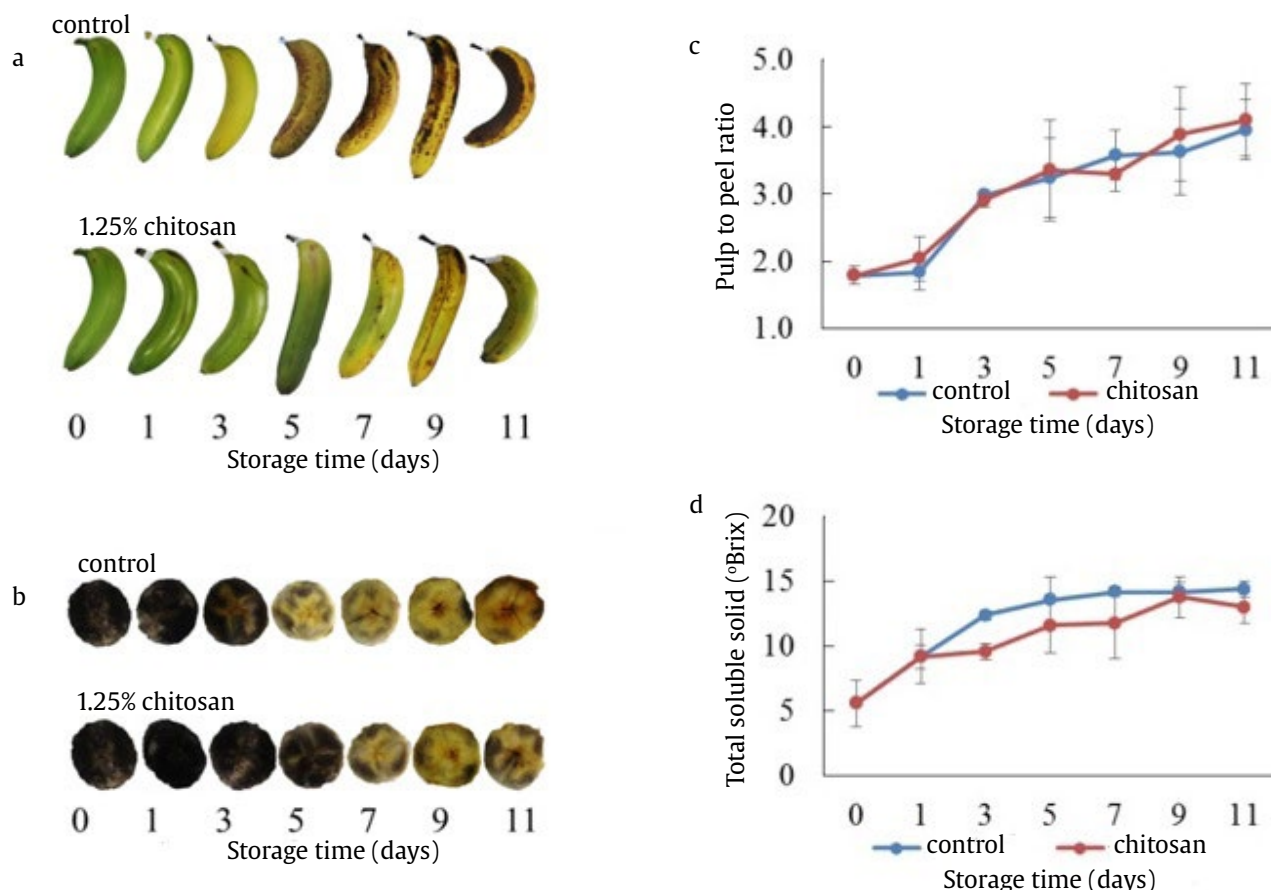


Figure 2. Physicochemical characteristics of uncoated and coated bananas with 1.25% chitosan solution during ripening from day 0 to 11. (a) Color changes of peel (b) Starch conversion pattern into sugars confirmed by using iodine reaction (c) Pulp to peel ratio based on weight. Blue: uncoated, Red: chitosan-coated 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

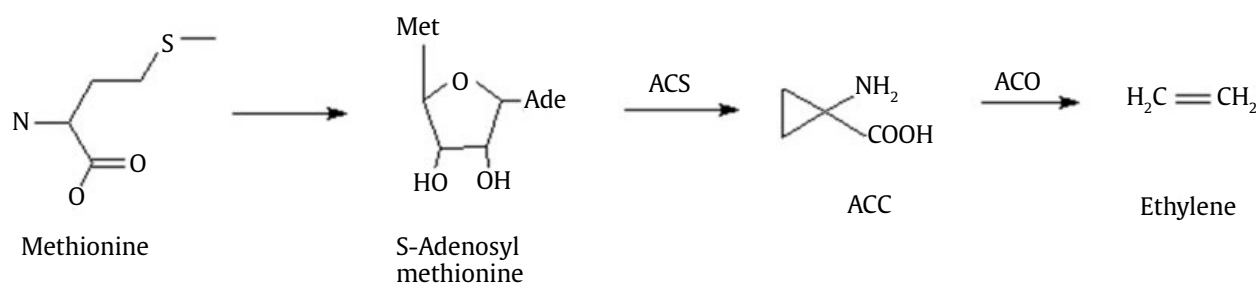


Figure 3. The ethylene biosynthesis pathway in plants in general. Ethylene is produced from methionine, which is first converted to S-adenosyl methionine (AdoMet). The next two steps are the conversion of AdoMet to ACC by ACS and the oxidative cleavage of ACC by ACO to form ethylene. (Met: Methionine,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

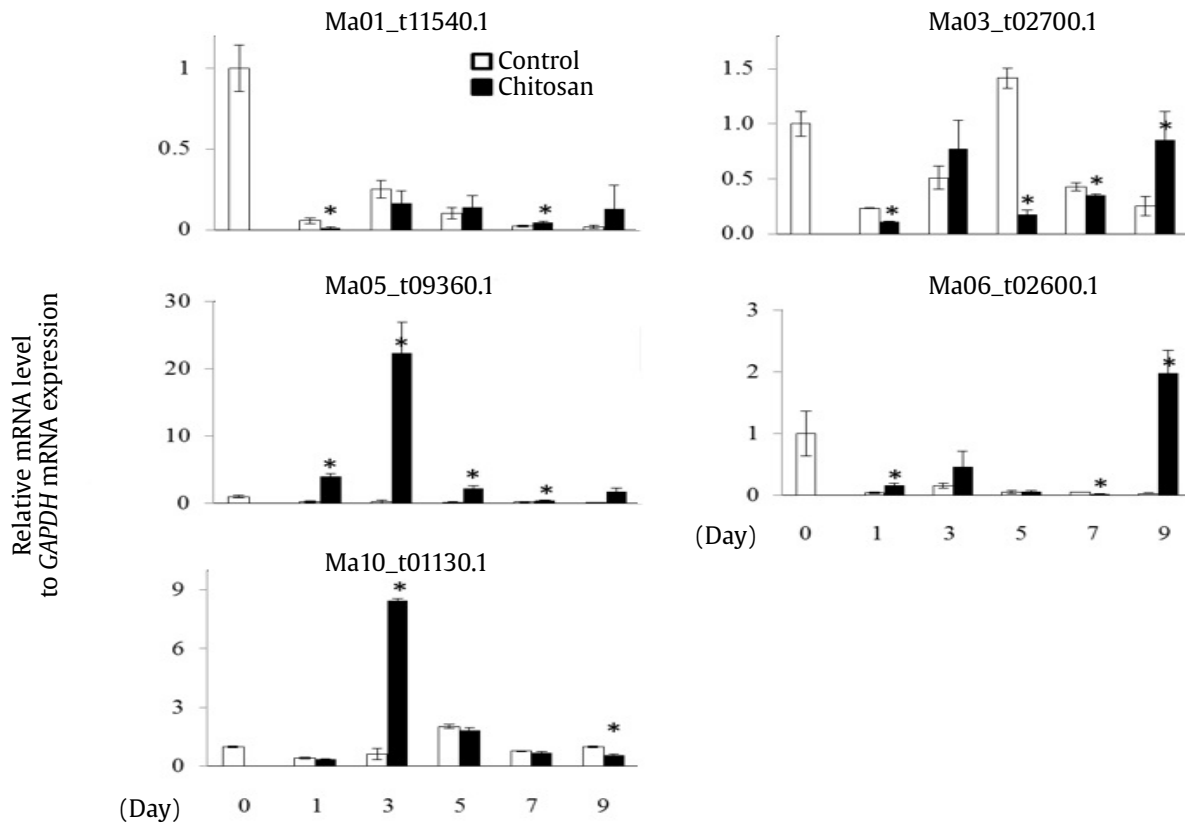


Figure 4. mRNA expression level of each ACO gene. Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from both uncoated and chitosan-coated banana fruit tissue during ripening on day 0, 1, 3, 5, 7 and 9. Each relative transcript abundance was normalized by MaGADPH 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.  $n = 3$

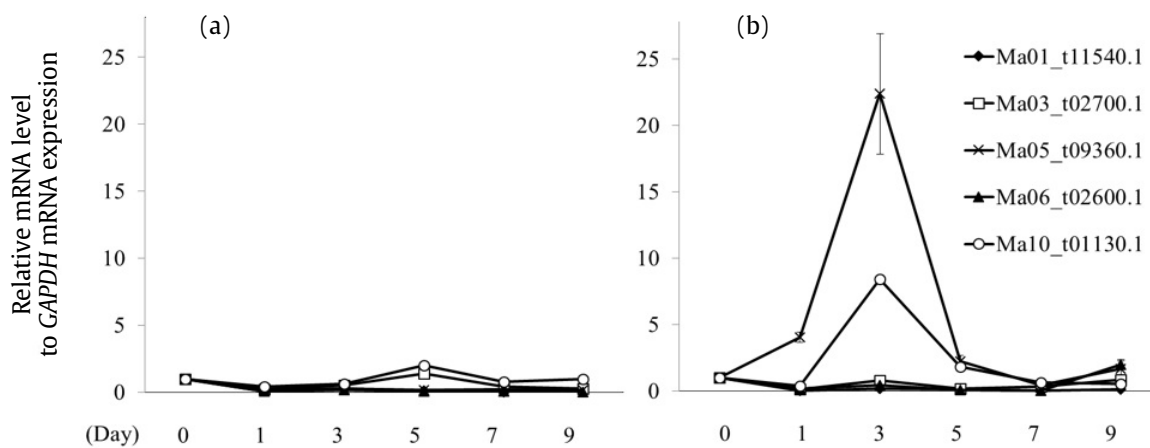


Figure 5. mRNA expression level of each ACO gene in uncoated banana (a) and chitosan-coated banana (b). Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from fruit tissue during ripening on day 0, 1, 3, 5, 7, and 9. Each relative transcript abundance was normalized 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<sub>2</sub> and high level of CO<sub>2</sub> 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<sub>2</sub> and CO<sub>2</sub>. Low level of O<sub>2</sub> and high level of CO<sub>2</sub> can inhibit the activities of enzymes involved in hydrolysis of starch (Maqbool *et al.* 2011). Furthermore, that O<sub>2</sub> and CO<sub>2</sub> 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<sub>2</sub> penetration (Jianglian and Shaoying 2013) and ACO needs O<sub>2</sub> 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<sub>2</sub>.

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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## References

- Ahmad S *et al.* 2001. Effect of Reduced O<sub>2</sub> and Increased CO<sub>2</sub> (Controlled Atmosphere Storage) On The Ripening and Quality of Ethylene Treated Banana Fruit. *Int J Agri Biol* 3(4).
- Alexander L., Grierson D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of Experimental Botany* 53(377):2039-2055.
- Asif MK *et al.* 2004. Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biology* 14:316.
- Banks NH. 1984. Some effects of TAL Pro-Long coating on ripening bananas. *J Expt Bot* 35:127-137.
- Burg SP, Burg EA. 1962. Role of ethylene in fruit ripening. *Plant Physiol* 37:179-189.
- Chien P *et al.* 2007. Effects of Edible Chitosan Coating on Quality and Shelf Life of Sliced Mango Fruit. *Journal of Food Engineering* 78:225-229.
- Dadzie BK, Orchard JE. 1997. Routine Post Harvest Screening of Banana/Plantain Hybrids: Criteria and Methods. *INIBAP Technical Guidelines 2*. Rome, Italy:International Plant Genetic Resources Institute.
- Dong H *et al.* 2004. Effects Of Chitosan Coating on Quality And Shelf Life of Peeled Litchi Fruit. *Journal of Food Engineering* 64:355-358.
- Dwivany FM *et al.* 2016. 'Raja Bulu' banana MaACS1 and MaACO1 gene expression during postharvest storage. *Acta Hort (ISHS)* 1120:111-114.
- Eshghi S *et al.* 2014. Effect of Nanochitosan-Based Coating With and Without Copper Loaded on Physicochemical and Bioactive Components of Fresh Strawberry Fruit (*Fragaria x ananassa* Duchesne) During Storage. *Food Bioprocess Technol* 7:2397-2409.
- Handayani RD, Dwivany FM. 2014. Analysis of MaACS2, a stress-inducible ACC Synthase Gene in *Musa acuminata* AAA Group Cultivar Pisang Ambon. *J Math Fund Sci* 46(2):194-203.
- Hubert O, Didier Mbéguié-A-Mbéguié. 2012. Expression patterns of ethylene biosynthesis genes from bananas during fruit ripening and in relationship with finger drop. *AoB PLANTS*, pls041; doi:10.1093/aobpla/pls041
- Jiang Y, Li Y. 2000. Effect of Chitosan Coating on Postharvest Life and Quality of Longan Fruit. *Food Chemistry* 73:139-143.
- Jianglian D, Shaoying S. 2013. Application of Chitosan Based Coating in Fruit and Vegetable Preservation: A Review. *Journal of Food Processing & Technology* 4 (227). doi:10.4172/2157-7110.1000227
- Karikari SK *et al.* 1979. Changes During The Respiratory Climacteric in Ripening Plantain Fruits. *Scientia Horticulturae* 10:369-376.
- Karmawan LU *et al.* 2009. Isolation of MA-ACS Gene Family and Expression Study of MA-ACS1 Gene in *Musa acuminata* cultivar Pisang Ambon Lumut. *Hayati of Bioscience* 16(1):35-39.
- Kende H. 1993. Ethylene Biosynthesis, *Annual Review Plant Physiology & Plant. Molecular Biology* 44:283-307.
- Kudachikar VB *et al.* 2011. Effect of Modified Atmosphere Packaging On Quality and Shelf Life of 'Robusta' Banana (*Musa sp.*) Stored at Low Temperature. *J Food Sci Technol* 48(3):319-324.
- Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2<sup>-ΔΔCT</sup> Method. *Methods* 25:402-408.

- Luo Y, Wang Q. 2013. Recent Advances of Chitosan and Its Derivatives for Novel Applications in Food Science. *J Food Processing & Beverages* 1(1):13.
- Maqbool M *et al.* 2011. Effect of a Novel Edible Composite Coating Based on Gum Arabic and Chitosan on Biochemical and Physiological Responses of Banana Fruits during Cold Storage. *J Agric Food Chem* 59:5474–5482.
- Nisperos MO, Baldwin EA. 1988. Effect of two types of edible films on tomato fruit ripening. *Proc Fla State Hort Soc* 101:217-220.
- Pratiwi A *et al.* 2015., Effect of Chitosan Coating and Bamboo FSC (Fruit Storage Chamber) to Expand Banana Shelf Life. *AIP Conference Proceedings* 1677, 100005.
- Song L, Liu D. 2015. Ethylene and plant responses to phosphate deficiency. *Front Plant Sci* 6:796.
- Sorrentino AG *et al.* 2007. Potential Perspectives of Bio-Nanocomposites for Food Packaging Applications. *Trends in Food Science & Technology* 18:84-95.

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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 biodegradable 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).

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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°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 0°Brix 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	CACGAACGGCAAGTA- CAAGA	CTGTTACCGTGGCCT- TCATT
Ma03_t02700.1 (XM_009392868.1)	chr03:1835668- 1839116	ACO-like	CGGTCATCGATTCTC- CAAG	TCGGAGCTGACCTTCT- TCAC
Ma05_t09360.1 (XM_009402256.1)	chr05:6781198- 6784233	ACO1-like	TCGACTGGGAGAC- CACCTAC	GAGGCGCAAATGTCT- TCTTC
Ma06_t02600.1 (XM_009404647.1)	chr06:2004016- 2005240	ACO1-like	GGAGTGATGGAG- GAGGTGAA	GATGGCGGTAGAA- GAAGCTG
Ma10_t01130.1 (XM_009422017.1)	chr10:4027373- 4031606	ACO homolog 3-like	GAGGATGGGGAGTCT- GAGTG	TCCAGGGTTGAAGAA- GGTTG

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

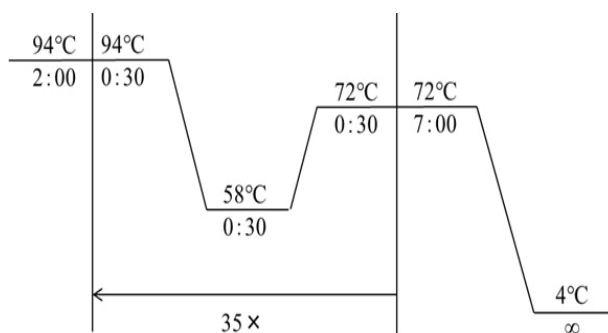


Figure 1. Cycle of PCR

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'-AGCAGCCTTGTCCTGTCA-3' (Handayani and Dwivany, 2012). The relative expression level was calculated using obtained Cq value (quantification cycle) by  $2^{-\Delta\Delta C_t}$  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

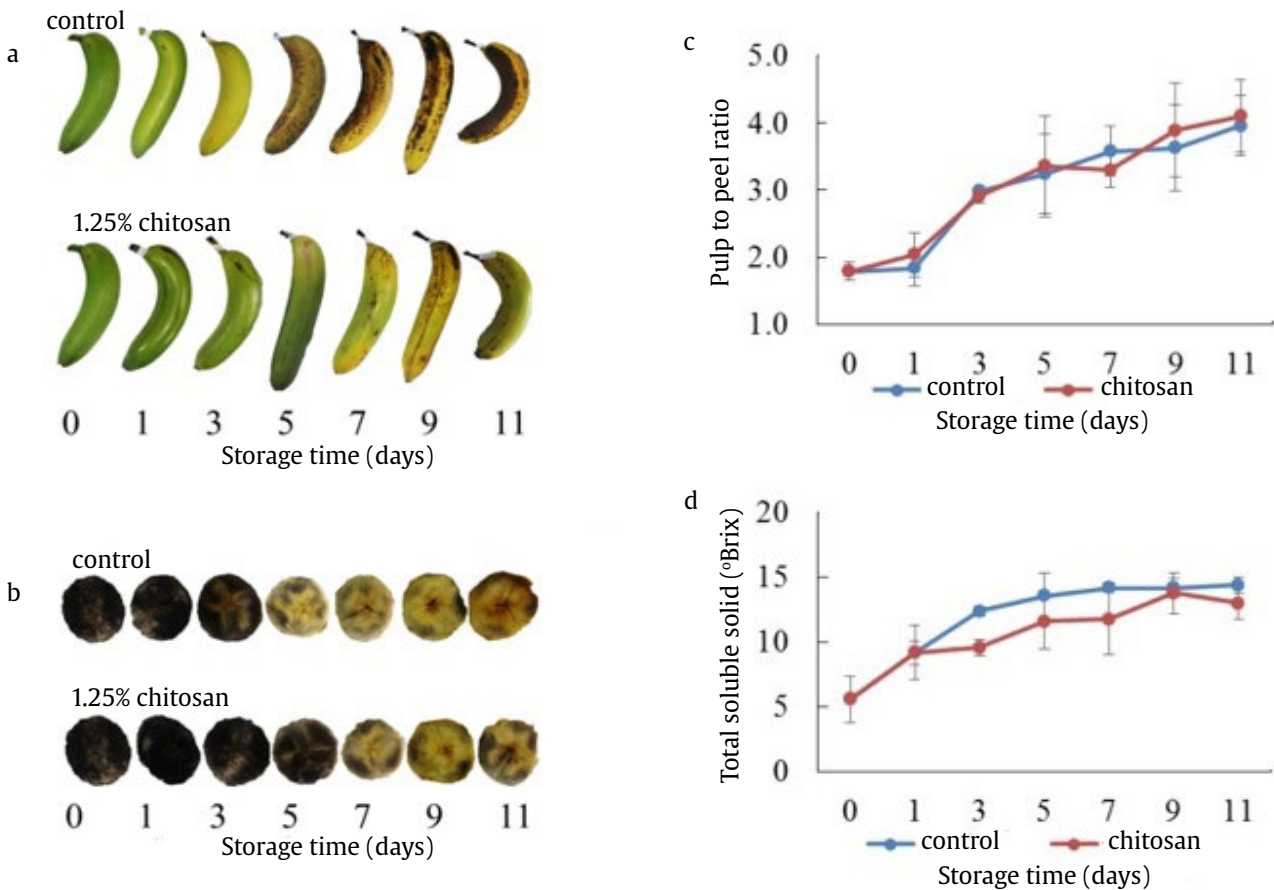


Figure 2. Physicochemical characteristics of uncoated and coated bananas with 1.25% chitosan solution during ripening from day 0 to 11. (a) Color changes of peel (b) Starch conversion pattern into sugars confirmed by using iodine reaction (c) Pulp to peel ratio based on weight. Blue: uncoated, Red: chitosan-coated 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

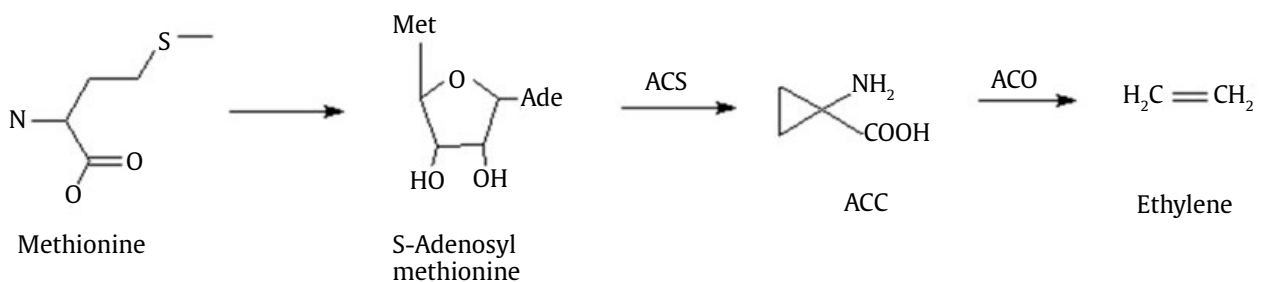


Figure 3. The ethylene biosynthesis pathway in plants in general. Ethylene is produced from methionine, which is first converted to S-adenosyl methionine (AdoMet). The next two steps are the conversion of AdoMet to ACC by ACS and the oxidative cleavage of ACC by ACO to form ethylene. (Met: Methionine,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

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#### 4. Discussion

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

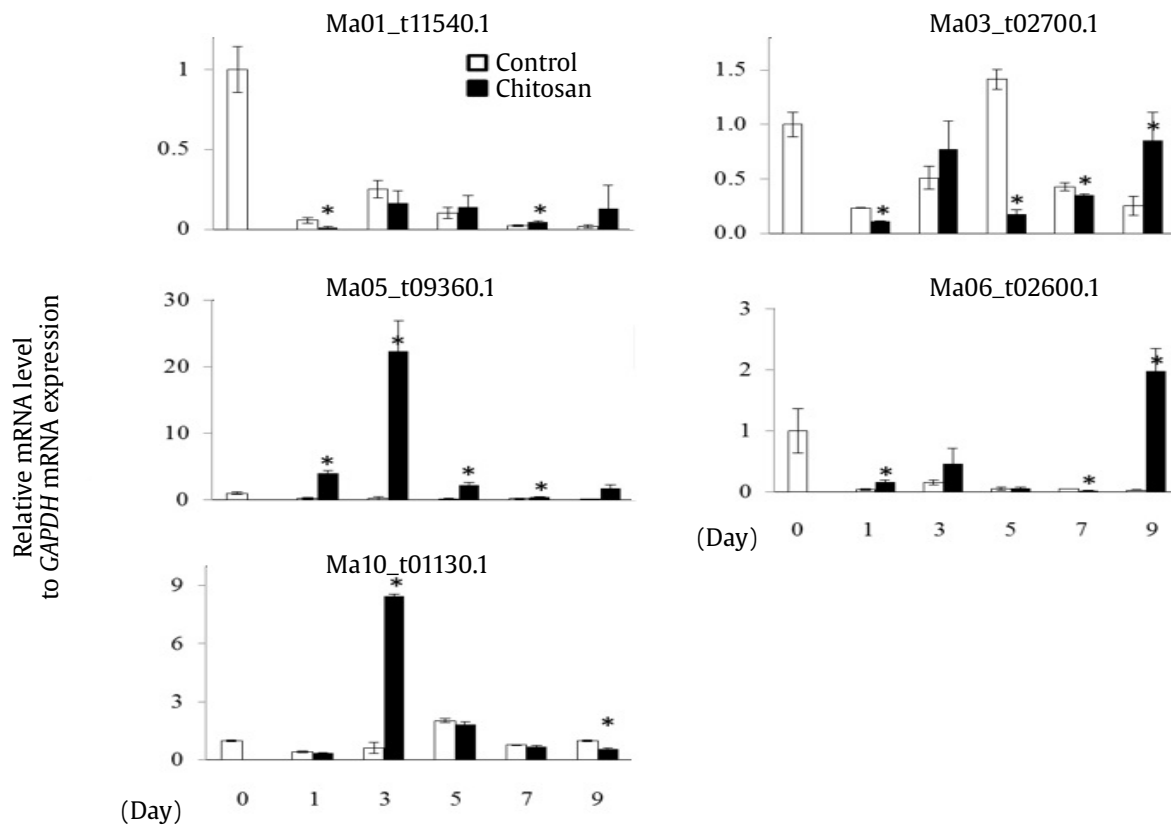


Figure 4. mRNA expression level of each ACO gene. Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from both uncoated and chitosan-coated banana fruit tissue during ripening on day 0, 1, 3, 5, 7 and 9. Each relative transcript abundance was normalized by *MaGAPDH* 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.  $n = 3$

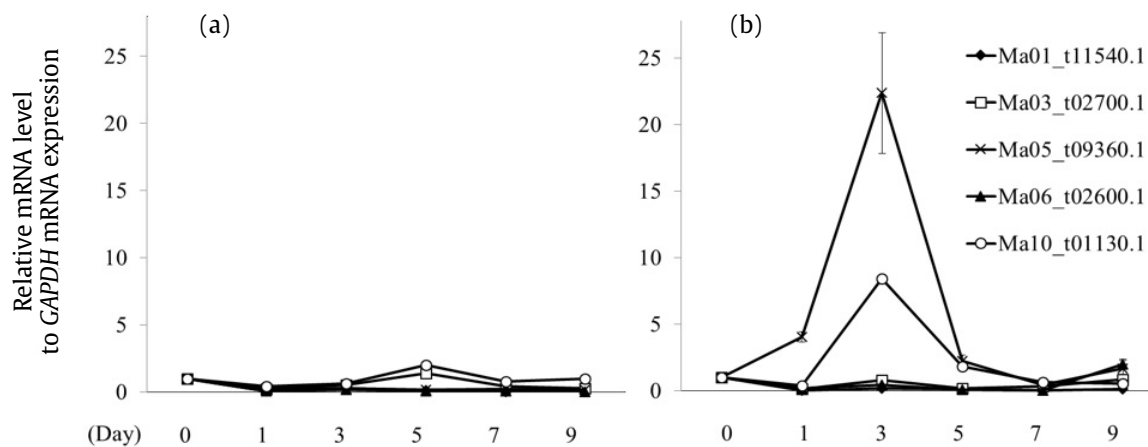


Figure 5. mRNA expression level of each ACO gene in uncoated banana (a) and chitosan-coated banana (b). Quantitative real-time PCR was carried out using cDNA synthesized from total RNA derived from fruit tissue during ripening on day 0, 1, 3, 5, 7, and 9. Each relative transcript abundance was normalized by *MaGAPDH* 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<sub>2</sub> and high level of CO<sub>2</sub> 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<sub>2</sub> and CO<sub>2</sub>. Low level of O<sub>2</sub> and high level of CO<sub>2</sub> can inhibit the activities of enzymes involved in hydrolysis of starch (Maqbool *et al.* 2011). Furthermore, that O<sub>2</sub> and CO<sub>2</sub> 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<sub>2</sub> penetration (Jianglian and Shaoying 2013) and ACO needs O<sub>2</sub> 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<sub>2</sub>.

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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## References

- Ahmad S *et al.* 2001. Effect of Reduced O<sub>2</sub> and Increased CO<sub>2</sub> (Controlled Atmosphere Storage) On The Ripening and Quality of Ethylene Treated Banana Fruit. *Int J Agri Biol* 3(4).
- Alexander L., Grierson D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of Experimental Botany* 53(377):2039-2055.
- Asif MK *et al.* 2004. Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biology* 14:316.
- Banks NH. 1984. Some effects of TAL Pro-Long coating on ripening bananas. *J Expt Bot* 35:127-137.
- Burg SP, Burg EA. 1962. Role of ethylene in fruit ripening. *Plant Physiol* 37:179-189.
- Chien P *et al.* 2007. Effects of Edible Chitosan Coating on Quality and Shelf Life of Sliced Mango Fruit. *Journal of Food Engineering* 78:225-229.
- Dadzie BK, Orchard JE. 1997. Routine Post Harvest Screening of Banana/Plantain Hybrids: Criteria and Methods. *INIBAP Technical Guidelines 2*. Rome, Italy:International Plant Genetic Resources Institute.
- Dong H *et al.* 2004. Effects Of Chitosan Coating on Quality And Shelf Life of Peeled Litchi Fruit. *Journal of Food Engineering* 64:355-358.
- Dwivany FM *et al.* 2016. 'Raja Bulu' banana MaACS1 and MaACO1 gene expression during postharvest storage. *Acta Hort (ISHS)* 1120:111-114.
- Eshghi S *et al.* 2014. Effect of Nanochitosan-Based Coating With and Without Copper Loaded on Physicochemical and Bioactive Components of Fresh Strawberry Fruit (*Fragaria x ananassa* Duchesne) During Storage. *Food Bioprocess Technol* 7:2397-2409.
- Handayani RD, Dwivany FM. 2014. Analysis of MaACS2, a stress-inducible ACC Synthase Gene in *Musa acuminata* AAA Group Cultivar Pisang Ambon, *J Math Fund Sci* 46(2):194-203.
- Hubert O, Didier Mbéguié-A-Mbéguié. 2012. Expression patterns of ethylene biosynthesis genes from bananas during fruit ripening and in relationship with finger drop. *Aob PLANTS*, pls041; doi:10.1093/aobpla/pls041
- Jiang Y, Li Y. 2000. Effect of Chitosan Coating on Postharvest Life and Quality of Longan Fruit. *Food Chemistry* 73:139-143.
- Jianglian D, Shaoying S. 2013. Application of Chitosan Based Coating in Fruit and Vegetable Preservation: A Review. *Journal of Food Processing & Technology* 4 (227). doi:10.4172/2157-7110.1000227
- Karikari SK *et al.* 1979. Changes During The Respiratory Climacteric in Ripening Plantain Fruits. *Scientia Horticulturae* 10:369-376.
- Karmawan LU *et al.* 2009. Isolation of MA-ACS Gene Family and Expression Study of MA-ACS1 Gene in *Musa acuminata* cultivar Pisang Ambon Lumut. *Hayati of Bioscience* 16(1):35-39.
- Kende H. 1993. Ethylene Biosynthesis, *Annual Review Plant Physiology & Plant. Molecular Biology* 44:283-307.
- Kudachikar VB *et al.* 2011. Effect of Modified Atmosphere Packaging On Quality and Shelf Life of 'Robusta' Banana (*Musa* sp.) Stored at Low Temperature. *J Food Sci Technol* 48(3):319-324.
- Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR

- and the 2- $\Delta\Delta\text{CT}$  Method. *Methods* 25:402–408.
- Luo Y, Wang Q. 2013. Recent Advances of Chitosan and Its Derivatives for Novel Applications in Food Science. *J Food Processing & Beverages* 1(1):13.
- Maqbool M *et al.* 2011. Effect of a Novel Edible Composite Coating Based on Gum Arabic and Chitosan on Biochemical and Physiological Responses of Banana Fruits during Cold Storage. *J Agric Food Chem* 59:5474–5482.
- Nisperos MO, Baldwin EA. 1988. Effect of two types of edible films on tomato fruit ripening. *Proc Fla State Hort Soc* 101:217-220.
- Pratiwi A *et al.* 2015., Effect of Chitosan Coating and Bamboo FSC (Fruit Storage Chamber) to Expand Banana Shelf Life. *AIP Conference Proceedings* 1677, 100005.
- Song L, Liu D. 2015. Ethylene and plant responses to phosphate deficiency. *Front Plant Sci* 6:796.
- Sorrentino AG *et al.* 2007. Potential Perspectives of Bio-Nanocomposites for Food Packaging Applications. *Trends in Food Science & Technology* 18:84-95.