Effect of Ethanol on ACC Synthase Activity and Gene Expression in Ripening Banana

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Postharvest application of 35~90% ethanol is reported to remove astringency and improve sweetness of banana. In order to determine the specific pattern and localization of gene expression, banana fruits were treated with 60% ethanol and control and then the total RNA extracted from the banana was hybridized with the RNA probe of ACC synthase gene. The highest ACC synthase activity was observed at 6 days after the ethanol treatment, while the accumulation of *MA-ACS1* mRNA (encoding banana ACC synthase during ripening) was observed in 9 days after the same treatment. In this experiment, the date of highest ethylene production coincided with the date of highest ACC synthase activity. The expression of ACC synthase gene is considered to occur firstly in pulp and then it occur in the peel on the basis of the signal intensity by Northern blot analysis.

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In banana fruit, ethanol did not induce a rapid and immediate increase in respiration in contrast to ethylene¹⁾. However, the respiratory peak of ethanol-treated fruits was observed earlier than that of the control fruits. Although ethylene is commercially used to ripen banana, postharvest application of 35~90% ethanol is reported to remove astringency and improve sweetness^{2),3)}. The fruit ripening is related to respiratory behavior and ethylene production during the later stages of maturation. The biosynthetic pathway of ethylene in higher plants was well characterized and ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.4.3) were identified. ACC synthase catalyzed the conversion of *S*-adenosylmethionine (SAM) to ACC and is regarded as the rate-limiting step⁴⁾.

Expression of ACC synthase gene has been investigated in many fruits, such as tomatoes^{5),6)}, apple⁷⁾, pumpkin⁸⁾, banana⁹⁾ and so on. ACC synthase is known to be encoded by a multigene family¹⁰⁾ and *MA-ACS1*, *ACS2* and *ACS3* among the family were already investigated in ethylene treated banana⁹⁾. The *MA-ACS1* and *MA-ACS2* mRNAs correlated with the ethylene production of ripening, wounding and sliced banana, respectively but the *MA-ACS3* showed low level of expression in banana⁹⁾. There is no work relating ACC synthase gene expression with ethanol-treated banana and

this prompted as to construct *MA-ACS1* probe and check the expression.

In order to determine the specific expression in 60% ethanol-treated and control banana, the total RNA was hybridized with the *MA-ACS1* mRNA probe which was synthesized from the banana cDNA of ACC synthase obtained from DDBJ database (AF 080258¹¹⁾).

Materials and Methods

1. Plant materials

Green 'Cavendish' bananas were purchased from a local wholesale market. The hands of banana were separated into individual fingers and distributed to 60% ethanol-treated and control groups. Each group was assigned to 3 jars. Six fingers (approximately 900 g) per jar were kept in 6 ℓ glass jars and sprayed with 10 ml of water (control) and 60% ethanol for 24 hr. The jars were immediately covered with perforated plastic film and were stored at 25°C. Peel color was monitored daily and scored according to an index of 1 (green) to 8 (yellow, surface covered with large senescent spots)¹²⁾. After treatment, three samples were selected randomly in the jars after the elapse of 0, 3, 6, 9 and 12 days. The fruit was cut horizontally into 3 portions and the central portion was divided into peel and pulp. Five mm thick pulps

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and 2 cm peels were frozen with liquid nitrogen and stored at -80°C until needed for RNA extraction.

2. Measurement of respiration (CO_2) and ethylene production

Each jar containing 12 fingers was kept sealed for 2 hr at 25°C . CO_2 in the headspace of the jar was measured by a gas chromatograph (GC) equipped with thermal conductivity detector (Shimazu GC-8 AIT).

From each treated-fruit before freezing with liquid nitrogen, 2 g of peel or pulp discs in capped test tube were incubated in a water bath shaker for 1 hr at 25°C . After incubation, the capped tube was transferred in the ice bath and ethylene produced in the head space of the tube was determined by gas chromatograph. Ethylene gas was also analyzed using the same GC model fitted with a flame ionization detector (FID) and all measuring conditions were the same as the previous paper¹³.

3. ACC synthase and ACC oxidase activity assays

Frozen tissue (2 g) was added in liquid nitrogen and was ground in a cooled mortar and pestle with 4 ml of extraction buffer (400 mM K-phosphate pH 8.5, 10 μM pyridoxal 5'-phosphate (PLP), 0.5% mercaptoethanol and 20% (W/V) glycerol). The resulting homogenate was filtered through 4 layers Kimwipe and the filtrate was charged on a dry Sephadex G-25 column (1.5 i.d. $\times$ 7 cm) prepared by centrifuging at 3,000 r.p.m for 3 min at 4°C just before use. The elute solution recentrifuged at the same condition was concentrated to 1/3 of its original volume by polyethylene glycol 6,000. The solution (2 ml) was applied to a Sephadex G-25 fine column (1.5 i.d. $\times$ 6 cm) equilibrated with 10 mM K-phosphate buffer (pH 8.5). The elute solution was used for the enzyme assay, according to the method described by LIZADA and YANG¹⁴. To 0.4 ml of the elute solution, 0.2 ml of 50 mM Hepes-KOH buffer containing 10 μM PLP and 200 μM SAM was added, and afterward it was incubated at 30°C for 15 min, followed by mixing with 400 μl of stopping reagent (Bleach-saturated NaOH (2 : 1, V/V) and 10 mM HgCl_2 in the ratio of 1 : 3 (V/V)). A blank experiment was carried out using ice bath instead of 30°C and the other conditions were the same described above. After immediately putting a stopper, the tube was shaken vigorously for 15 sec and kept in the ice bath for 3 min. Ethylene produced in the head space (1 ml) of capped tubes

after 1 hr incubation was determined by the same GC described above. The assay for ACC oxidase was made as in the previous paper¹⁵.

4. RNA extraction and cloning of ACC synthase and ACC oxidase genes

The frozen tissue was pulverized to a fine powder with a mortar and pestle. Total RNA was extracted by the hot borate method¹⁶. The total RNA was directly used for cDNA synthesis without the isolation of poly (A)⁺ RNA. To isolate ACC synthase fragments, degenerate oligonucleotide primers homologous to conserved regions of ACC synthase were synthesized. The degenerate oligonucleotide primers for PCR were synthesized to two amino acid domains conserved in bananas ACC synthase genes, for the sense primer (5'-CAACGCCWTATTAYCCGGGAT-3'), and the anti-sense primer (5'-TGGCRAARCACACCTGAACC-3'), respectively. The first strand cDNAs, synthesized from 2 μg of total RNA isolated from ethanol-treated fruits, were performed with a SuperScriptTM Preamplification System for First Strand cDNA (BRL, Tokyo, Japan) containing RT. The primers were amplified by PCR. The parameters for PCR were 30 cycles of heating at 94°C for 45 s, at 55°C for 30 s and at 72°C for 90 s. The *Sal* I and *Not* I restriction site sequences were also included at the 5'-end of both sense and antisense primers, to facilitate cloning of PCR product. The PCR products were cloned into the pSPORT 1 vector (BRL, Tokyo, Japan). The cloning of ACC oxidase was performed in a similar manner as in the previous paper¹⁵.

5. Preparation of the digoxigenin (DIG) -UTP-labeled RNA probe

The cloned RT-PCR product including the coding region of ACC synthase gene was cleaved by *Not* I and *Sal* I from the pSPORT 1 vector that had been amplified in *Escherichia coli* (DH-5 α) and it was purified by gel electrophoresis and recovered. Antisense DIG-labeled RNA probes were prepared using cloned RT-PCR product, and the DIG RNA Labeling kit (Boehringer Mannheim) according to the manufacturer's instruction. The RNA probe of ACC oxidase was produced in a similar manner as in the previous paper¹⁵.

6. Northern blot hybridization

Ten μg of total RNA was subjected to electrophoresis on a 1.0% agarose gel that contained 0.66 M formaldehyde and transferred to

Hybond-N⁺ (Amersham) by capillary action with 20 × SSC, according to manufacturer's instruction. After drying the membrane, the RNAs were fixed with UV. The membrane was preincubated in 5 × SSPE, 5 × Denhart's solution and 0.5% SDS at 60 °C for 3 h and then probed with the gene-specific antisense DIG-labeled RNA probe at 60 °C for 24 h. The membrane was washed once with 2 × SSPE containing 0.1% SDS for 10 min at room temperature, once with 1 × SSPE plus 0.1% SDS for 15 min at 65 °C and twice with 0.2 × SSPE containing 0.1% SDS for 15 min each at 65 °C. After allowing sheep anti-DIG Fab¹⁷ fragments conjugated to alkaline phosphatase to bind to the DIG-labeled probe, the excess antibody was removed. Signals were detected by color reaction using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium salt as the substrate. RNA samples were ensured by measuring UV spectro meter (UV-1200, Shimazu, Japan) and hybridizing an internal control gene, banana actin cDNA. In the case of ACC oxidase, the procedure for Northern blot hybridization was made as in the previous paper¹⁵.

Fig. 1 Changes in peel color development (A) and respiration rate (B) of ethanol-treated banana fruits stored at 25 °C

Each point represents the mean of 3 replicates.

Vertical bars indicate the standard error when larger than symbol.

Results and Discussion

1. Changes in peel color development, respiration rate and ethylene production

Fig. 1 (A) shows the peel color development in the 60% ethanol-treated and control bananas. The development of the yellow color of the peel was hastened by the application of 60% ethanol. Ripening of the fruit to color stage 5 (yellow with green tips) took about 7 or 8 days in control fruits, and 5 or 6 days in 60% ethanol-treated fruits. The fruits ripeness in this experiment reached 2 ~ 3 days faster than the previous paper¹⁵.

Ethanol-treated banana after harvest showed more uniform ripening¹⁸ (means consistent sugar, organic acid contents and color) than control banana, whereas ethylene-treated banana was superior to the ethanol-treated banana in relation to the uniform ripening¹⁸.

The climacteric maximum corresponding to respiration maximum was observed at 9 and 6 days after treatment in control and 60% ethanol-treated fruits, respectively (Fig. 1 (B)).

Fig. 2 shows ethylene production in the peel and

Fig. 2 Changes in the level of ethylene production in control (A) and 60% ethanol-treated banana (B) fruits stored at 25 °C

Each point represents the mean of 3 replicates.

Vertical bars indicate the standard error when larger than symbol.

pulp of ethanol-treated and control bananas. The highest ethylene production of control and 60% ethanol-treated fruit was at 9 and 6 days, respectively. In ethanol-treated and control fruits, the ethylene showed higher production in peel than that in pulp.

The climacteric maximum (Fig.1(B)) was the same day of the highest ethylene production in the 60% ethanol-treated (Fig.2 (B)) and the control fruit (Fig.2 (A)), respectively. The climacteric maximum in the ethanol-treated fruit was accelerated by 1 day in comparison with the previous paper¹⁵⁾ since the higher ethylene production was caused by the faster ripening.

2. Changes in ACC synthase and ACC oxidase

Figs.3 and 4 show changes in ACC synthase

and ACC oxidase activities of pulp (A) and their expressions in Northern blot analysis on ethanol-treated (B, B') and control peel (C) and pulp (C') of banana fruits, respectively. The ACC synthase activity of ethanol-treated pulp was observed between 3 and 6 days after treatment and peaked on 6 days, whereas the maximum activity of control pulp was observed at 3 days behind the maximum peak of the ethanol-treated pulp. Due to existence of disturbing substances in the peel, the enzyme activity was not calculated in the peel for both treatments. As the ethylene production was observed in the peel for both treatments, ACC synthase is considered to be present in the peel. The highest ACC synthase activity in the pulp was observed at 9 and 6

Fig. 3 Changes in ACC synthase activity (A) in pulp and its expression in Northern blot analysis on ethanol-treated (B) and control (C) banana peel and pulp stored at 25°C

- (A) : ACC synthase activities of banana pulp. Each point represents the mean of 3 replicates. Vertical bars indicate the standard error when larger than symbol. ACC synthase activities of banana peel could not be measured due to the existence of disturbing substances.
- (B), (B') : Expression of ACC synthase genes in ethanol-treated banana fruits, peel and pulp, respectively.
- (C), (C') : Expression of ACC synthase genes in control banana fruits, peel and pulp, respectively.
- (D), (D') : Expression of Actin genes in ethanol-treated banana fruits, peel and pulp, respectively. (Data not shown in control) Each lane contained 10 μ g total RNA. Blots were hybridized with DIG labeled *MA-ACS 1* mRNA probe. *MA-actin* was used as internal standard of the blot intensity.

Fig. 4 Changes in ACC oxidase activity (A) in pulp and its expression in Northern blot analysis on ethanol-treated (B) and control (C) banana peel and pulp stored at 25°C

- (A) : ACC oxidase activities of banana pulp. Each point represents the mean of 3 replicates. Vertical bars indicate the standard error when larger than symbol. ACC oxidase activities of banana peel could not be measured due to the existence of disturbing substances.
- (B), (B') : Expression of ACC oxidase genes in ethanol-treated banana fruits, peel and pulp, respectively.
- (C), (C') : Expression of ACC oxidase genes in control banana fruits, peel and pulp, respectively.
- (D), (D') : Expression of Actin genes in ethanol-treated banana fruits, peel and pulp, respectively. (Data not shown in control) Each lane contained 10 μ g total RNA. Blots were hybridized with DIG labeled *MA-ACO 1* mRNA probe. *MA-actin* was used as internal standard of the blot intensity.

days after treatment in control and 60% ethanol-treated fruits, respectively and it coincided with the highest ethylene production.

As shown in Fig.4, the highest ACC oxidase activity in the ethanol treated pulp was observed at 9 days after treatment. The highest ACC synthase activity appeared ahead of the highest ACC oxidase activity.

3. Expression of ACC synthase and ACC oxidase genes

The behavior of ACC synthase activity in the pulp (Fig.3 (A)) paralleled that of the ethylene production in control (Fig.2 (A)) and 60% ethanol-treated pulp (Fig.2 (B)). The accumulation of *MA-ACS 1* mRNA in the ethanol-treated pulp showed an increasing trend until 9 days and declined in 12 days, whereas that in control reached a high level in 12 days. The *MA-ACS 1* gene showed higher message level in the ethanol-treated than control. The same trend was observed in the peel but the accumulation of *MA-ACS* level was lower than the pulp. The accumulation of *MA-ACS 1* mRNA in the ethanol-treated pulp was observed ahead of that in the peel, whereas that of *MA-ACO 1* mRNA (Fig.4 (B')) was hastened earlier 3 days than that of *MA-ACS 1* mRNA. Similar observation that ACC oxidase gene showed faster expression than ACC synthase gene had been already reported by LIU, *et al.*⁹⁾ on the basis of Northern blot analysis. The ethylene production requires both ACC synthase and oxidase, especially the synthase is regarded as the rate-limiting enzyme⁴⁾.

According to LIU *et al.*⁹⁾, only *MA-ACS 1* mRNA was detected in ripening banana. *MA-ACS 1* mRNA coincided with ACC synthase activity in the ethylene-treated banana. Among the members of *MA-ACS* multigene family, *MA-ACS 2* mRNA was reported to be induced by wounding banana, whereas the transcript of *MA-ACS 3* mRNA was reported to be undetected by Northern analysis⁹⁾. Therefore, *MA-ACS 2* and *MA-ACS 3* mRNA are considered not to coincide with ACC synthase activity in our report. However, the problem that the expression of *MA-ACS 1* mRNA did not parallel ACC synthase activity is unknown at this time.

After all, we found that the highest accumulation date of *MA-ACS 1* mRNA was observed at 9 days when the highest ACC synthase activity was observed at 6 days after the ethanol treatment. The expression of ACC synthase gene is considered

to occur firstly in pulp and then it occur in the peel on the basis of Northern blot results.

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**追熟バナナ果実のACC合成酵素活性と
遺伝子発現におけるエタノールの効果**

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収穫後に35~90%のエタノールでバナナ果実を処理すると渋味の除去と甘味の増すことが報告されている。遺伝子発現の特異的なパターンと局在を調べるために、バナナ果実を60%エタノール処理とコントロール処理し、ついでバナナから抽出された全RNAをACC合成酵素遺伝子のプローブとハイブリダイゼーションさせた。

最も高いACC合成酵素活性は、エタノール処理後6日目に観察され、一方MA-ACS1のmRNA(バナナのACC合成酵素遺伝子をコードしている)は同様の処理後9日目であった。本報告で、最も高いエチレン生成日は最も高いACC合成酵素活性日に一致していた。

ノーザン分析のシグナル強度に基づくとACC合成酵素遺伝子の発現がはじめに果肉で起こり、ついで果皮で起こると考えられる。

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