Temporal Relationship between Ester Biosynthesis and Ripening Events in Bananas

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ABSTRACT. The temporal relationship between changes in ethylene production, respiration, skin color, chlorophyll fluorescence, volatile ester biosynthesis, and expression of *ACC oxidase* (*ACO*) and *alcohol acyl-CoA transferase* (*AAT*) in ripening banana (*Musa* L. spp., AAA group, Cavendish subgroup, 'Valery') fruit was investigated at 22 °C. Ethylene production rose to a peak a few hours after the onset of its logarithmic phase; the peak in production coincided with maximal *ACO* expression. The respiratory rise began as ethylene production increased, reaching its maximum ≈30 to 40 hours after ethylene production had peaked. Green skin coloration and photochemical efficiency, as measured by chlorophyll fluorescence, declined simultaneously after the peak in ethylene biosynthesis. Natural ester biosynthesis began 40 to 50 hours after the peak in ethylene biosynthesis, reaching maximal levels 3 to 4 days later. While *AAT* expression was detected throughout, the maximum level of expression was detected at the onset of natural ester biosynthesis. The synthesis of unsaturated esters began 100 hours after the peak in ethylene and increased with time, suggesting the lipoxygenase pathway be a source of ester substrates late in ripening. Incorporation of exogenously supplied ester precursors (1-butanol, butyric acid, and 3-methyl-1-butanol) in the vapor phase into esters was maturity-dependent. The pattern of induced esters and expression data for *AAT* suggested that banana fruit have the capacity to synthesize esters over 100 hours before the onset of natural ester biosynthesis. We hypothesize the primary limiting factor in ester biosynthesis before natural production is precursor availability, but, as ester biosynthesis is engaged, the activity of alcohol acyl-CoA transferase the enzyme responsible for ester biosynthesis, exerts a major influence.

A temporal relationship has been established between ethylene production and several physiological changes including those related to skin color (Peacock, 1972; Seymour et al., 1987), respiration, and starch conversion (Beaudry et al., 1989). Although ripening is associated with a decline in chlorophyll fluorescence (Smillie et al., 1987), the temporal relationship between chlorophyll fluorescence and natural ethylene synthesis has not been described. Similarly, aroma biosynthesis is known to be associated with ethylene synthesis and action (Golding et al., 1998; Peacock, 1972); however, its temporal relationship to ethylene production and other ripeness stage indices such as respiration, color, and chlorophyll fluorescence has not been studied for individual fruit.

The aroma of banana fruit (*Musa* spp. AAA group, Cavendish subgroup, 'Valery') is an important eating quality criteria that influences consumer acceptability. More than 250 volatile components have been identified in banana (Macku and Jennings, 1987; Shiota, 1993; Tressl et al., 1970). By combining analytical chemistry with sensory measurements, several volatile compounds have been determined to be 'impact compounds', which contribute significantly to aroma and confer typical aroma perceptions. Penten-2-one and the 3-methylbutyl and 2-methylpropyl esters of acetate and butanoate, have been found to be the major contributors to banana fruit aroma (Berger et al., 1986). The temporal relationship between endogenous ethylene production,

respiration, and volatile biosynthesis has not been reported.

Esters are derived from amino acid and fatty acid metabolism (Myers et al., 1970; Tressl et al., 1970). Alcohol acyl-CoA transferase (*AAT*, EC 2.3.1.84) combines alcohols and CoA derivatives of short to medium chain length fatty acids to form esters. *AAT* has been identified and partially purified in ripe banana fruit (Harada et al., 1985; Ueda and Ogata, 1977) and its gene identified originally in strawberry (*Fragaria ×ananassa* Duchesne) (Aharoni et al., 2000) and subsequently in banana (Aharoni et al., 2001). *AAT* activity increases during banana fruit ripening for fruit induced to ripen by exposure to exogenous ethylene (Harada et al., 1985). In the study by Harada et al. (1985), *in vivo* *AAT* activity was estimated by supplying tissue samples with 3-methyl-1-butanol and measuring the increase in the production of 3-methylbutyl acetate. Gene expression for *AAT* has not been reported for banana, but has been shown to increase for strawberry as they ripen (Aharoni et al., 2000).

The primary objective of this study was to characterize the temporal relationship between changes in ripening indices and aroma-related volatile biosynthesis. Particular emphasis was placed on temporal changes in the capacity of banana fruit to synthesize esters *in vivo* from exogenously supplied alcohols, acids, and their combination. In our study, whole banana fruit were enclosed in respiratory chambers at room temperature (22 °C) and changes in skin hue, chlorophyll fluorescence, CO₂ production, and C₂H₄ biosynthesis were determined relative to the synthesis of aroma-related volatile compounds. In addition, whole fruit and tissue plugs of peel and pulp were excised at three stages of fruit development (before the ethylene climacteric, at the peak in ethylene synthesis, and at the peak in ester formation) and their capacity to incorporate alcohols and acids into esters was determined by measuring net changes in ester production. We focused on the metabolism of 3-methyl-1-butanol, 1-butanol, and butyrate and the important aroma impact compounds 3-methylbutyl acetate and 3-methylbutyl butanoate. Finally, to understand the relation between changes in the production of ethylene and esters, we performed Northern analysis for *ACC oxidase* (*ACO*) and *AAT*, respectively.

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Materials and Methods

PLANT MATERIAL. Mature green banana fruit (*Musa* spp. AAA group, Cavendish subgroup, 'Valery') were obtained from a local ripening facility on the day of their receipt at the facility and before exposure to ethylene. At receipt, fruit temperature was $\approx 13^{\circ}\text{C}$. Fruit were transferred immediately to the laboratory and warmed to ambient temperature $22 \pm 0.5^{\circ}\text{C}$.

RESPIROMETRY STUDY. Upon arrival at the laboratory, fruit were weighed and fruit uniform in coloration and free from obvious defects were selected for the study. Ten banana fruit were placed individually in 2.1-L glass chambers at 22°C the day of arrival and ventilated with purified air at a flow rate of 25 to 30 $\text{mL}\cdot\text{min}^{-1}$. Air in the chambers was humidified by directing incoming air across the surface of water in a humidification chamber upstream of the respirometer. Water content of the atmosphere was not measured, but condensation that occurred on the sides of the jar suggested the relative humidity was elevated. No shriveling or discoloration of the peel surface was noted. Gas lines entering and exiting the glass chambers were composed of Teflon, which has an extremely low sorptivity for most aroma volatiles.

Sampling for CO_2 and ethylene production was carried out two to four times daily, depending on the stage of development, with more frequent sampling occurring before and during the development of the respiratory and ethylene climacterics. Respiration and ethylene measurement were as described below. Volatile concentrations in the exit gas streams were sampled at the same time as ethylene and CO_2 and measurements were made as described below. Chlorophyll fluorescence was measured once daily on fruit removed temporarily from the jars after gas exchange measurements were made. Skin color was measured after fluorescence measurements were taken and at the same positions on the stem end, middle, and blossom end of the fruit.

This study was conducted twice. The data from each study were very similar. Data from the second replication are presented. Of the 10 initial fruit, four fruit for which the peak in ethylene synthesis was captured were tracked throughout the ripening period. The production of aroma-related volatiles was determined at each measurement period as indicated below. All data were temporally normalized relative to the peak in ethylene biosynthesis such that time 0 represents the peak in ethylene synthesis.

FEEDING STUDIES. Production of CO_2 and ethylene was measured on two additional lots of fruit as for the respirometry study. One of these lots of fruit was used to determine in vivo activity of AAT in whole fruit and the second lot was used to determine AAT activity in excised fruit tissue. Fruit were selected for analysis according to their ripeness stage as judged by ethylene and CO_2 biosynthesis. Stages for analysis were preclimacteric stage (A), the climacteric peak in ethylene production (B), and near the maximum in ester production (C). For both lots, the fruit reached the peak in ethylene synthesis ≈ 100 h after samples were taken to represent the preclimacteric stage.

In the whole fruit feeding study, three fruit were selected at each stage of development, removed from the respirometry chambers, and placed into identical 2.1-L chambers fitted with a Teflon-lined half-hole septum. The capacity of whole fruit to synthesize esters from alcohol and acid precursors was determined relative to nontreated controls by supplying banana fruit with vapor of 3-methyl-1-butanol, butyric acid, and their combination.

Vapors of 3-methyl-1-butanol and butyric acid were generated by injecting 5 μL of the pure liquids into the 2.1-L chambers in which the fruit were sealed. After 3 h incubation, incorporation of

3-methyl-1-butanol into 3-methylbutyl acetate and 3-methylbutyl butanoate, butyric acid into butyl butanoate and 3-methyl butanoate, and the mixture of 3-methyl-1-butanol and butyric acid into 3-methylbutyl acetate, 3-methylbutyl butanoate, and butyl butanoate were measured. Butyl acetate, which should not be affected by the precursors provided, was also quantified as a control. Data are presented as the amount ($\text{nmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) of ester formation in excess of that formed by control fruit. Three fruit at each stage were used for measurement.

For the excised tissue study, ester forming capacity was determined for peel and pulp samples taken from fruit at ripening stages A, B and C. Tissue samples were obtained by cutting tissue into plugs ($\approx 0.25\text{ cm}^2$) and placing them on filter paper, which was placed in a 25-mL glass vial fitted with a Mininert valve (Altech Assoc., Inc, Deerfield, Ill). Filter paper had been immersed into 1.0 mL of a 0.1 mM 2-(N-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.5) solution. For the precursor incubation, the buffer contained 0.1 mM 3-methyl-1-butanol or 1-butanol or their combination and buffer alone was used as control. Volatiles in the headspace were sampled after 4 h incubation as described below and analyzed by gas chromatography (GC)/mass spectrometry (MS). Incorporation of 3-methyl-1-butanol into 3-methylbutyl acetate, and 1-butanol incorporation into butyl acetate and butyl butanoate were measured and the net production ($\text{nmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) relative to controls reported.

RESPIRATION AND ETHYLENE MEASUREMENTS. Respiration was calculated from CO_2 production by the fruit. A 250-L gas sample was withdrawn from the outlet of the 2.1-L chamber and analyzed for CO_2 using an infrared gas analyzer (model 225-MK3; Analytical Dev. Co., Hoddesdon, U.K.). To measure ethylene production, a second gas sample (1 mL) was removed from the exit line and assayed for ethylene using a gas chromatograph (Carle Series 100 AGC, Hach Co., Loveland, Colo.). A certified gas standard (Matheson Gas Products, Chicago, Ill.) was used to calculate ethylene and CO_2 concentrations.

CHLOROPHYLL FLUORESCENCE. Fluorescence measurements were made in a laboratory that was dimmed to dark-adapt the tissue. Banana fruit were placed at the end of the fiber optic light guide of a pulse-modulated fluorometer (model OS-500; Opti-Science, Tyngsboro, Mass.). The fluorometer was operated in the Fv/Fm mode and fluorescence was measured as described previously for ripening apple [*Malus sylvestris* (L) Mill var *domestica* (Borkh.) Mansf] (Song et al., 1997a). Measurements were made at the stem end, the middle, and the blossom end of each of fruit. Chlorophyll fluorescence was measured daily.

SKIN COLOR. Skin color of banana fruit was measured as lightness, chroma, and hue angle with a color meter (Chromameter 300; Minolta Camera Co., Osaka, Japan). Color readings were made at the same positions as the chlorophyll fluorescence measurements on each fruit. Only hue angle data are reported.

VOLATILE ANALYSIS. Sampling of volatile compounds was accomplished using solid phase microextraction (SPME) as described previously (Song et al., 1997b). The SPME fiber, coated with 65 μm -thick polydimethylsiloxane/divinylbenzene, was inserted into the exit line of the respirometer and volatiles were absorbed for 4 min. Volatile separation and detection were carried out by GC/time-of-flight MS as described previously (Song et al., 1997b). The minimum level of detection was $<1\text{ nL}\cdot\text{L}^{-1}$ in the gas phase for all compounds evaluated.

For the respirometry study, the data represent the concentration of the volatiles in the exit gas stream of the chambers. Volatile quantities are reported as the response of the mass spectrometer,

which was calculated as the total ion count (TIC) for each compound.

For the feeding studies, 1-butanol, butyl acetate, 3-methyl-1-butanol, 3-methylbutyl acetate, 3-methylbutyl butanoate, butyl butanoate, and hexyl acetate were quantified relative to a gas standard made from authenticated compounds (Aldrich Co., Milwaukee, Wis.). For standard preparation, compounds were mixed as liquids in equal volume aliquots. Three microliters of the mixture was placed in a glass 4.4-L volumetric flask fitted with a ground glass stopper containing a gas-tight Mininert valve (Altech Assoc., Inc.). The flask was held at 22 °C for more than 4 h, permitting the compounds to vaporize fully.

RNA EXTRACTION AND ANALYSIS. Total RNA was extracted from the banana pulp using the cetyltrimethylammonium bromide (CTAB)/NaCl method (Chang, 1993) as modified by Asif et al. (2000) using a polysaccharide precipitation step after LiCl precipitation.

Northern analysis was essentially as described by Sambrook et al. (1989). Total RNA isolated from different stages of ripening banana was separated by electrophoresis on 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and 1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA). Fifteen micrograms of total RNA of each sample was loaded in each lane of the agarose gel. The gel was blotted overnight by capillary transfer onto a nylon membrane (Hybond N, Amersham) in 20× SSPE and fixed by incubating at 80 °C for 3 h. The RNA blots were prehybridized at 42 °C using formamide buffer containing Denhardt's reagent and denatured salmon sperm DNA.

Fig. 1. Changes in the respiration rate, ethylene biosynthesis, chlorophyll fluorescence, hue, and total volatile production as represented by the total ion count (TIC) of the mass spectrometer response for all volatiles exclusive of CO₂ during ripening of whole bananas at 22 °C from 100 h before to 270 h after the peak in ethylene synthesis. A, B, and C are time points corresponding to a preclimacteric stage, the ethylene peak, and the ester production maximum, respectively (n = 3); vertical bars represent 1 SD. Inset: Correlation between skin hue angle and measurements of chlorophyll fluorescence Fv/Fm. $Fv/Fm = -1.46 + 0.0186 \times (\text{hue angle})$, $r^2 = 0.92$.

The cDNA probes, banana AAT (gi:10187178) and heterologous ACO (gi:232041) obtained from apple were labeled essentially as described by Sambrook et al. (1989) using the random-primed DNA labeling kit (Gibco-BRL, Rockville, MD) with [³²P]dCTP. Labeled plasmid inserts were purified by Sephadex-50 columns. Labeled probes were eluted from the column using three column volumes of Tris-EDTA buffer (10 mM Tris and 1 mM EDTA). The eluate was denatured by heating to 100 °C and added to the prehybridization mix. The membranes were hybridized overnight at 42 °C.

Following the hybridization, the membranes were washed twice at 22 °C with 3× SSPE buffer with 0.1% (w/v) SDS for 30 min and with 1× SSPE buffer with 0.1% (w/v) SDS for 30 min and with 0.1× SSPE with 0.1% (w/v) SDS for 15 min. The blots were dried with Whatman 3M paper and exposed to x-ray films with intensifying screens at -80 °C for overnight to 3 d based on the intensity of the signal.

Results

RESPIROMETRY STUDY. The onset of climacteric ethylene production occurred between 3 and 7 d after fruit were placed in the respirometers. Ethylene production remained low for ~100 h, increasing rapidly thereafter (Fig. 1). The peak in ethylene production took place ~24 to 48 h after increased production was first detected, undergoing a phase of logarithmic increase of about 8 h in duration and reaching a maximum at time 0 (time point B on Fig. 1). The respiration rate increased as ethylene production increased initially, but did not peak until ~48 h after the peak in ethylene synthesis. After reaching its peak in production, ethylene declined

Fig. 2. Changes in the TIC for 3-methylbutyl acetate (3MBA, ▲), 3-methyl-1-butanol (3MB-ol, ▼), and their ratio (●) with ripening for whole bananas held at 22 °C from 100 h before to 270 h after the peak in ethylene synthesis. Data are the mean mass spectrometer responses for volatiles from four fruit; vertical bars represent 1 SD.

to 1/3 its maximal level in ≈ 30 to 40 h. Respiratory CO_2 production also declined after reaching its maximum, but more slowly than ethylene and to a lesser extent.

Skin color started to change rapidly when ethylene biosynthesis peaked (Fig. 1). Hue angle decreased from 120° to 80° over a 5 d period as visible color changed from dark green to full yellow. The change in skin color coincided with a decrease in photochemical quantum efficiency as measured by a decline in chlorophyll fluorescence (Fig. 1). The decrease in chlorophyll fluorescence began coincident with the ethylene peak; the drop in F_v/F_m for the stem end tending to occur a few hours earlier than for the middle and blossom end of the fruit (data not presented). Much lower chlorophyll fluorescence was measured as the fruit reached the full yellow stage with light brown spots ≈ 100 to 120 h after the peak in ethylene biosynthesis. A linear relationship ($r^2 = 0.9$) existed between chlorophyll fluorescence and hue angle (Fig. 1, inset).

During the early stages of ripening (time points A and B on Fig. 1), ester production was not detectable. The onset of ester biosynthesis lagged behind the peak in ethylene biosynthesis by 40 to 50 h, occurring concurrently or soon after the rate of respiration reached

its maximum. The maximal rate of volatile production was reached ≈ 150 h after the peak in ethylene biosynthesis (time point C on Fig. 1).

Trends were apparent in the relative production of several esters and their precursors. The branched-chain alcohol 3-methyl-1-butanol and its ester product, 3-methylbutyl acetate, were detected simultaneously for each fruit (Fig. 2). However, 3-methyl-1-butanol was relatively more abundant in the early stages of ester production. The relative abundance of the alcohol to the ester declined ≈ 20 -fold over the course of the experiment.

Production of the ester 3-methylbutyl butanoate began at the same time or slightly ahead of 3-methylbutyl acetate. Earlier 3-methylbutyl butanoate production only occurred when butyrate production also occurred earlier than 3-methylbutyl acetate. The most abundant ester detected was 3-methylbutyl butanoate accounting for $\approx 1/4$ of the total MS response (Fig. 3). The temporal patterns of the relative abundance of 3-methyl-1-butanol and butyrate differed from one another, with the alcohol being relatively abundant early in ester synthesis and to a lesser extent at the end of the monitoring period.

Fig. 3. Changes in the TIC for 3-methylbutyl butanoate (3MBB, $\blacktriangle$), 3-methyl-1-butanol (3-MB-ol, $\blacktriangledown$), butanoic acid (B-ic, $\diamond$), the ratio of 3MB-ol to 3MBB ($\bullet$), the ratio of B-ic to 3MBB ($\circ$), and the ratio of 3-MB-ol to B-ic ($\blacksquare$) with ripening for whole bananas held at 22°C from 100 h before to 270 h after the peak in ethylene synthesis. Data are the mean mass spectrometer responses for volatiles from four fruit; vertical bars represent 1 sd.

Fig. 4. Changes in the TIC for 4-hexenyl butanoate (4HXB, $\blacktriangle$), 4-hexene-1-ol (4HX-ol, $\blacktriangledown$), butanoic acid (B-ic, $\diamond$), the ratio of 4HX-ol to 4HXB ($\bullet$), the ratio of B-ic to 4HXB ($\circ$), and the ratio of 4-HXB-ol to B-ic ($\blacksquare$) with ripening for whole bananas held at 22°C from 100 h before to 270 h after the peak in ethylene synthesis. Data are the mean mass spectrometer responses for volatiles from four fruit; vertical bars represent 1 sd.

Table 1. Net ester formation ($\text{nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \pm \text{SD}$) by whole bananas after incubation at different ripening stages with 3-methyl-1-butanol, butyric acid, and their combination for 3 h. A, B, and C refer to time points corresponding to a preclimacteric stage, the ethylene peak, and the ester production maximum, respectively, as indicated in Fig. 1. Data are net ester biosynthesis relative to nontreated fruit at the same stage of development, $n = 3$; only those data where the net synthesis was greater than the SD are reported.

Esters	3-Methyl-1-butanol			Butyric acid			3-Methyl-1-butanol + butyric acid		
	A	B	C	A	B	C	A	B	C
Butyl acetate	0	0	0	0.1 ± 0.1	0	0	0	0.6 ± 0.04	0.1 ± 0.1
3-Methylbutyl acetate	0	13.5 ± 2.3	25.1 ± 3.5	0.4 ± 0.2	0.4 ± 0.1	0	0.8 ± 0.5	0.4 ± 0.03	23.2 ± 3.4
Butyl butanoate	0	0	0	0.3 ± 0.1	1.6 ± 0.4	21.1 ± 1.9	0	0	34.0 ± 2.5
3-Methylbutyl butanoate	0	0	0.5 ± 0.2	0	2.9 ± 0.6	8.1 ± 3.1	3.6 ± 1.0	8.3 ± 0.6	20.3 ± 2.3

Table 2. Net ester formation ($\text{nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \pm \text{SD}$) after feeding the excised peel and pulp tissue of banana at different ripening stages with 0.1 mM solutions of 3-methyl-1-butanol and 1-butanol for 4 h. A, B, and C refer to time points corresponding to a preclimacteric stage, the ethylene peak, and the ester production maximum, respectively, as shown in Fig. 1. Data are net ester biosynthesis relative to nontreated tissue at the same stage of development ($n=3$). Only those data where the net synthesis was greater than the SD are reported.

Esters	Peel			Pulp		
	A	B	C	A	B	C
3-Methyl-1-butanol						
Butyl acetate	0	0	0	0	0.12 ± 0.05	0.44 ± 0.2
3-Methylbutyl acetate	0	0.4 ± 0.20	3.1 ± 0.8	0	5.28 ± 1.30	4.71 ± 2.0
Butyl butanoate	0	0	0	0	0.07 ± 0.01	0.84 ± 0.3
1-Butanol						
Butyl acetate	0	0.05 ± 0.03	1.2 ± 0.7	0	0.12 ± 0.03	1.34 ± 0.6
3-Methylbutyl acetate	0	0	0	0	0.07 ± 0.01	0.11 ± 0.1
Butyl butanoate	0	0	1.1 ± 0.7	0	0.18 ± 0.10	1.24 ± 1.0

Butyl butanoate production was first detected simultaneously with or just after the production of 3-methylbutyl acetate (data not presented). The MS response for butyl butanoate was $<1/10$ that of 3-methylbutyl acetate. The precursor alcohol, 1-butanol, was most abundant relative to butyl butanoate during the earliest stages of ester production, whereas the acid, butanoate, was relatively most abundant when ester production was high.

The unsaturated ester 4-hexenyl butanoate was first detected ≈ 100 h after the ethylene peak (at about the full yellow stage) and increased markedly thereafter (Fig. 4). During the early phase of the production of this ester, butanoic acid was its most abundant relative to the ester, with the ratio declining approximately 50-fold as the fruit aged. The alcohol precursor, 4-hexenyl-1-ol, was of constant abundance relative to the ester throughout the study period. The abundance of the alcohol relative to butyrate increased as fruit senesced.

When the abundance of the esters 3-methylbutyl acetate, butyl butyrate, and 4-hexenyl butyrate were evaluated relative to 3-methyl butyl butanoate, it increased for each as fruit aged (data not presented). The relative abundance of butyl butyrate increased most rapidly during the first 20 h of ester synthesis (hours 50 to 70), the relative abundance of 3-methylbutyl acetate increased steadily throughout ripening, and the relative abundance of 4-hexenyl butyrate increased most rapidly 150 h after the peak in ethylene synthesis.

PRECURSOR FEEDING STUDIES FOR WHOLE FRUIT. In preclimacteric fruit, no ester production was detected (data not presented). At this same stage, there was no detectable formation of either 3-methylbutyl acetate or 3-methylbutyl butyrate from feeding whole fruit 3-methyl-1-butanol (Table 1). Similarly, application of butyric acid only marginally enhanced formation of butyl butanoate and resulted in no detectable increase in 3-methyl butanoate. However, when 3-methyl-1-butanol and butyric acid were provided together at the

preclimacteric stage, significant production of the ester 3-methylbutyl butanoate, and to a lesser extent, 3-methylbutyl acetate, occurred. At the peak in ethylene production, native ester formation was not detectable. Feeding the fruit vapors of 3-methyl-1-butanol, led to formation of 3-methylbutyl acetate and addition of butyrate vapors lead to the preferential production of butyl butanoate and 3-methylbutyl butanoate. In addition, there was a slight net increase in 3-methylbutyl acetate formation in response to butyrate feeding. When 3-methyl-1-butanol and butyric acid were provided together at timepoint B, the primary product was 3-methylbutyl butanoate, although there was a limited net production of butyl acetate and 3-methylbutyl acetate. About 100 h after the peak in ethylene biosynthesis and when native ester production was near its maximum (timepoint C on Fig. 1), feeding 3-methyl-1-butanol preferentially lead to a net increase in 3-methylbutyl acetate with a small net increase in 3-methylbutyl butanoate (Table 1). Feeding butyric acid at this stage lead to a net increase in butyl butanoate and 3-

Fig. 5. Changes in the expression of AAT and ACO at different stages of ripening as represented by hours relative to peak in ethylene production for bananas. The 28S ribosomal RNA was an internal standard for evaluating loading of total RNA in each lane.

methylbutyl butanoate. The net increases in ester production due to precursor feeding were greater at timepoint C than at the peak in ethylene production (timepoint B). When 3-methyl-1-butanol and butyrate were applied together during the stage of rapid ester biosynthesis, they markedly enhanced production of 3-methylbutyl butanoate, 3-methylbutyl acetate, and butyl butanoate.

PRECURSOR FEEDING STUDIES FOR EXCISED FRUIT TISSUES. No enhancement of ester formation by 3-methyl-1-butanol or 1-butanol for peel or pulp tissue was detected at the preclimacteric stage (Table 2). At the climacteric peak in ethylene biosynthesis, ester biosynthesis from nontreated peel and pulp tissues was essentially nil (data not presented). However, for peel tissue, there was a slight stimulation in production of 3-methylbutyl acetate by 3-methyl-1-butanol and a marginally detectable increase in butyl acetate and butyl butanoate by 1-butanol. For pulp, feeding with 3-methyl-1-butanol slightly stimulated butyl acetate and butyl butanoate, but markedly stimulated 3-methylbutyl acetate synthesis. When the pulp was fed 1-butanol, there was a slight stimulation in the production of 3-methylbutyl acetate and a somewhat greater increase in butyl acetate and butyl butanoate. At the stage coinciding with maximal ester biosynthesis (timepoint C on Fig. 1), the pattern of ester induction was qualitatively similar to that at the climacteric peak; 3-methyl-1-butanol caused a marked increase in 3-methylbutyl acetate for peel and the pulp, and resulted in a slight increase in butyl acetate and butyl butanoate production by the pulp (Table 2). Addition of 1-butanol brought about a marked enhancement of butyl acetate and butyl butanoate production for peel and pulp tissues and a slight promotion of 3-methylbutyl acetate in the pulp. Relative to the climacteric peak, induction of 3-methylbutyl acetate by 3-methyl-1-butanol increased in the peel, but not in the pulp. However, 1-butanol feeding brought about stimulation of butyl acetate and butyl butanoate for both tissues when these two stages are compared. Incorporation of 3-methyl-1-butanol into esters was greater than that of 1-butanol at each developmental stage.

GENE EXPRESSION FOR AAT AND ACO. AAT expression was detected in fruit of all stages evaluated from 100 h before the ethylene peak to 260 h after (Fig. 5). AAT expression was minimal in green banana 100 h before the ethylene peak and maximal expression occurred 50 to 100 h after ethylene peak. The mRNA for AAT began to accumulate before the onset of aroma production. Expression declined after peak aroma production. ACO expression was also detected in all stages of ripening banana, peaking with maximal ethylene production. The level of ACO expression declined after the climacteric.

Discussion

Ripening-related changes in ethylene, respiration, and skin color measured in this study were consistent with previous findings on banana (Beaudry et al., 1989; Peacock, 1972). The decline in chlorophyll fluorescence (Fv/Fm) occurring with or just after the peak in endogenous ethylene synthesis is similar to the findings of Smillie et al. (1987) for fruit treated with exogenous ethylene. Furthermore, the high degree of correlation between color and Fv/Fm is similar to that reported previously for banana (Smillie et al., 1987) and for apples (Song, et al., 1997a). Smillie et al. (1987), reported the decline in Fv/Fm during ripening of banana fruit at 22 °C was a result of loss in chlorophyll content and a decrease in photosynthetic competency per unit chlorophyll. Unlike color measurement, which can be influenced by a number of pigments and events related to their production or loss (Seymour et al., 1987), chlorophyll fluorescence is the product of the response of a specific

class of pigments in a single type of organelle. The decline in fluorescence may serve as a useful index of chloroplast reorganization and fruit ripening and senescence.

The esters and other volatiles detected from ripening banana fruit in this study were the same as those found in previous investigations (De Pooter et al., 1983; Drawert et al., 1973; Macku and Jennings, 1987), however, the temporal relationship between ethylene production, respiration, and the onset of volatile biosynthesis has not been described previously. The fact that ester biosynthesis trailed the peak in ethylene biosynthesis by 40 to 50 h and lagged the peak in respiration by ≈20 h suggests that the ripening process is highly regulated throughout this period.

Stimulation of ester formation by feeding 3-methyl-1-butanol, butanol, and butyrate is consistent with previous studies demonstrating the incorporation of exogenously supplied alcohols and acids into these esters by AAT (DePooter et al., 1983; Knee and Hatfield, 1981; Kollmannsberger and Berger, 1992; Olias et al., 1995; Tressl and Drawert, 1973; Ueda and Ogata, 1977; Ueda et al., 1992). Our measurements of AAT activity in whole fruit were largely qualitative, given that there was no measure of the uptake of the supplied precursors nor a measure of their tissue concentration. Nevertheless, the approach used herein enables comparison of differing stages of development.

The appreciable stimulation of esters at the preclimacteric stage by the combination of 3-methyl-1-butanol and butyric acid, but not by either compound when applied separately, demonstrated all the machinery for ester formation from alcohol and acid substrates exists before initiation of natural ester formation. Incorporation of butyrate indicates butyric acid was activated by acyl CoA synthetase (EC 6.2.1.3) to form butyryl-CoA. Thus, it appears that substrate availability rather than ester-forming capacity limits ester production in preclimacteric bananas. Precursor feeding studies in preclimacteric harvested apple fruit (De Pooter et al., 1983; Knee and Hatfield, 1981; Song and Bangerth, 1994) also demonstrated that the supply of substrates seems to be the limiting factor, rather than the amount of AAT present before the onset of ripening.

As ethylene biosynthesis peaked, induction of esters in whole fruit and excised tissue by the addition alcohols or acids supplied separately demonstrated that ester biosynthesis could be induced. Presumably, the tissue was able to synthesize alcohols in response to an excess of acids and, conversely, synthesize acids in response to an excess of alcohols. Given that the fruit had the capacity to synthesize precursors of either class, but did not, suggests that precursor biosynthesis may have been down-regulated during the ethylene climacteric. The implication is that the initial increase in ester production may have resulted from a release from suppression of synthesis of either class of substrate. The increase in responsiveness of peel and pulp to exogenous substrates at the same stage of development suggests that the competency to synthesize esters progressed at the roughly the same rate for both tissue types.

When 3-methyl-1-butanol was supplied alone to whole fruit at the peak in ethylene synthesis, its preferential incorporation into the acetate ester rather than the butanoate ester suggests that acetyl-CoA was either more freely available than butyryl-CoA or was a better cosubstrate at this developmental. In support of the latter possibility, AAT from banana has been shown to have a very high preference (≈30:1) for acetyl-CoA relative to propyl- or butyryl-CoA (Olias, et al., 1995; Ueda et al., 1992).

The elevated abundance of the alcohols 3-methyl-1-butanol and butanol relative to their esters early in the ester production phase (hours 40 to 75 in this study) suggests that the factor most limiting to ester production during this developmental stage was not the

availability of the alcohol precursors, but may be the availability of the precursor to the acid portion of the ester molecule. The fact that 3-methylbutyl butanoate was produced earlier than 3-methylbutyl acetate only for those fruit for which butyrate was detected in the chamber headspace supports this contention. It is not clear whether it is the synthesis of the acid or the CoA derivative of the acid that would be limiting. However, in the precursor feeding experiment, feeding the butyric acid and the 3-methyl-1-butanol together resulted in butyrate ester formation by the preclimacteric banana.

The timing of the upsurge in esters suggests that by the time the volatiles synthesis was initialized, conversion of starch to sugars would have been 50% complete or more (Beaudry et al., 1989). Beaudry et al. (1989) determined that carbon conversion peaks at about the same time that respiration reaches its maximum and is declining rapidly 40 h after the peak in ethylene biosynthesis. As a result, a decline in the metabolic energy demand needed to convert starch to sugar would likely occur at this stage. Respiration, however, does not return to preclimacteric levels, suggesting the respiration rate may be in 'excess' relative to the energy demand. A rationale for a physiological link between the onset of ester synthesis and this potential excess respiratory capacity is not obvious.

The increasing rate of conversion of the acids, alcohols, and their mixture into esters as ripeness stage advanced is consistent with the finding by Harada et al. (1985) that AAT activity in cell-free extracts increases during fruit ripening. Similarly, Tressl and Drawert (1973) found that short and medium-chain ^{14}C acids are incorporated into esters by tissue disks at both climacteric and postclimacteric stages, but that incorporation was several times greater at the postclimacteric stage. Given the abundance of 3-methyl-1-butanol and butyrate in the headspace of the respirometry chambers in this study as ripeness advanced, AAT activity, rather than substrate availability, may have exerted the greater influence in ester formation as fruit became suitable for consumption. Aharoni et al. (2000) reported that AAT of banana has high activity with butanol and acetyl CoA but showed less activity with butyl CoA. Such specificity may enable different fruits to generate characteristic aroma profiles.

Data for expression of AAT reveal there was basal expression even in unripe green banana fruit and that expression increased as ripening proceeded. These data are consistent with the *in vivo* measures of AAT activity presented herein, which were low before ripening and increased as ripening progressed. Like banana, low expression of AAT was also found in white strawberry fruit followed by an increase as ripening and color change took place (Aharoni et al., 2000).

Data for the ACC oxidase gene expression were essentially similar to those reported by Liu et al. (1999). The reduction in ethylene production in the later stages of ripening, despite elevated gene expression of ACO and ACS was ascribed to limiting levels of the cofactors ascorbate and soluble iron in the later stages of fruit ripening. The peak in ACO expression preceded that of banana AAT. Ethylene may modulate, but not completely control AAT expression.

Appearance of unsaturated esters such as 4-hexenyl butyrate well after the onset of ester production, suggests that processes responsible for creating ester precursors continue to undergo developmental changes even during the latter stages of ripening and senescence. While the source of 4-hexen-1-ol has not been determined for banana, it is possible that it is a product of the oxidation of unsaturated fatty acids. The lipoxygenase pathway product, *cis*-3-hexenol and its respective acetate and butyrate esters underwent changes in concentration that were similar to 4-hexen-1-ol, 4-hexenyl acetate and 4-hexenyl butyrate (data not presented). The

presence of these unsaturated alcohols and their esters late in ripening suggests that membrane degradation may be occurring at these late stages. The appearance and accumulation of unsaturated volatiles coincided with the onset and the increase in browning of the peel. It is possible that the tissue breakdown associated with peel browning could be related to the generation of the unsaturated volatiles.

Collectively, the data in this study indicate that ester biosynthesis is influenced by substrate availability, AAT expression, and AAT activity levels in banana. The relative importance of these factors appears to change as ripening progresses, with substrate availability exerting the greater control before ester biosynthesis. This, and the tight temporal relationship of volatile production to other parameters of ripening during the natural ripening process suggest the process of aroma volatile formation is well regulated even into the latter stages of fruit ripening. Insight as to the controlling mechanisms may provide a means to enhance or better control fruit aromas.

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