Oleic Acid and Its Positional Isomer, cis-Vaccinic Acid, in the Appendix of Sauromatum guttatum during Anthesis

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One striking example of a thermogenic plant organ is provided by the inflorescence of Sauromatum guttatum (the voodoo lily). On D-day, the day of inflorescence opening, the Sauromatum appendix (a 20-cm-long, slender organ) becomes hot, reaching a temperature of 32°C (Skubatz et al., 1991). The heat facilitates the vaporization of odoriferous compounds that attract pollinators; the heat loss reaches a value of 1 mW/mg dry weight (Skubatz and Meeuse, 1993). The heat is generated by the mitochondria via an alternative, cyanide-resistant pathway. The activity of this pathway, which is present in the mitochondria of many plant species, does not produce ATP, and the energy is thus released as heat (Siedow and Moore, 1993).

One appendix was used for the analysis of fatty acids in the morning and evening of the same day. The developmental stage of the appendix was determined retroactively with respect to D-day. Sporophylls of thermogenic male cones of two cycad species (Encephalartos ferox and Dioon edule var angustifolium and var edule) were cut from plants grown outdoors and kept frozen until extraction was performed (Tang et al., 1987).

The inflorescences of Sauromatum guttatum were allowed to develop from corms under a 15-h/9-h light/dark cycle at 19°C in a growth chamber (Skubatz et al., 1991). One appendix was used for the analysis of fatty acids in the morning and evening of the same day. The developmental stage of the appendix was determined retroactively with respect to D-day. Sporophylls of thermogenic male cones of two cycad species (Encephalartos ferox and Dioon edule var angustifolium and var edule) were cut from plants grown outdoors and kept frozen until extraction was performed (Tang et al., 1987).

Materials and Methods

Plant Material and Growth Conditions

The inflorescences of Sauromatum guttatum were allowed to develop from corms under a 15-h/9-h light/dark cycle at 19°C in a growth chamber (Skubatz et al., 1991). One appendix was used for the analysis of fatty acids in the morning and evening of the same day. The developmental stage of the appendix was determined retroactively with respect to D-day. Sporophylls of thermogenic male cones of two cycad species (Encephalartos ferox and Dioon edule var angustifolium and var edule) were cut from plants grown outdoors and kept frozen until extraction was performed (Tang et al., 1987).

Chemicals and Reagents

Fatty acids and FAMES were purchased from Sigma. Methanolic 1 m HCl used in the transesterification of the fatty acids was purchased from Supelco (Bellefonte, PA).

Fatty Acid Extraction

Ten to 20 μg of tissue was extracted in 1 mL of methanolic 1 m HCl in the presence of the internal standard storage organelles of triacylglycerides, are depleted during the day of thermogenic activity, which is designated as D-day. Therefore, we have decided to determine the composition of total fatty acids in the appendix and in other organs of the inflorescence (Fig. 1), and in thermogenic male cones of two cycad species (Encephalartos ferox and Dioon edule var angustifolium and var edule) during development. We report here on changes in the levels of cis-vaccinic, oleic, and palmitoleic acids in the Sauromatum appendix during anthesis. cis-Vaccinic acid is present in the six other organs of the Sauromatum inflorescence and in the thermogenic sporophylls of the cycad species. But none of these organs shows the proportional change in the percentage of oleic and cis-vaccinic acids.

Abbreviations: D-day, day of inflorescence opening or of heat production; D−1, D−2, etc., days before D-day; D+1, D+2, etc., days after D-day; FAMES, fatty acid methyl esters; FTIR, Fourier-transform IR spectroscopy.

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The fatty acid profiles of various organs of the thermogenic inflorescence of Sauromatum guttatum and of the sporophylls of thermogenic male cones of two cycad species (Encephalartos ferox and Dioon edule var edule and var angustifolium) were determined by gas chromatography. During anthesis, palmitate (16:0), oleate (18:1 (9)), cis-vaccinate (18:1 (11)), and linoleate (18:2 (9, 12)) were the most abundant fatty acids in the Sauromatum appendix. cis-Vaccinic acid, a positional isomer of oleic acid, was identified by comparing its retention time on a gas chromatography column and its mass spectrum to an authentic compound. The percentage of oleic acid from total fatty acids dropped from about 9 in the morning 2 d before heat production to 6 in the morning 2 d before heat production. At this time, the percentage of cis-vaccinic acid increased from 3 to 11%, and then remained at this level until the inflorescence dried and died. Palmitoleic acid (16:1 (9)), the common precursor of cis-vaccinic acid, is a minor component of total fatty acids. In six other organs of the Sauromatum inflorescence including thermogenic organs, such as male flowers and lower spadix, palmitate, oleate, and linoleate were prevalent but cis-vaccinate was not. The thermogenic male cones of the two cycad species were rich in palmitic, oleic, and linolenic acids. The level of cis-vaccinic acid in these organs was less than 0.5%.

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Electron microscopic study of the Sauromatum appendix has revealed the presence of numerous lipid bodies in the cells (Skubatz et al., 1993). It appears that the lipid bodies,
triheptadecanoin (1,2,3-triheptadecanoyl glycerol, 50 μg/sample) at 80°C for 1 h. One milliliter of 10% KCl and 0.5 mL of hexane were added and FAMES was extracted into hexane three times. The hexane phase was then concentrated under nitrogen to 0.1 to 0.2 mL, and 1 to 2 μL of the solution was used for GC analysis. Fatty acids were also extracted from 1.5 g of tissue and methylated, and their absolute amount was determined by weight.

GC

Analysis of the FAMES composition of various extracts was performed using a Hewlett-Packard (Palo Alto, CA) 5890B gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard 7673A autosampler. A fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used, coated with the bonded stationary phase, Supelcowax 10 (Supelco), using helium as a carrier and makeup gas with a column flow of 1 mL min⁻¹. Samples were injected in the split mode (approximately 60:1) at a column temperature of 200°C. Upon injection the column oven temperature was immediately programmed linearly at 5°C min⁻¹ to 260°C and held for 4 min. The injector and detector temperatures were both maintained at 270°C.

GC/MS

GC/MS analysis of the oleic and cis-vaccinic methyl esters was carried out by comparing their retention times with those of a mixture of authentic fatty acyl methyl esters. Composition was reported as the percentage of the combined area of all peaks excluding the internal standard.

FTIR

FAMES were prepared from D-2 and D-1 appendices and dissolved in carbon disulfide at a concentration of 10 mg/mL. The IR absorption of the extracts was determined using a FTIR spectrometer (model 1460, Perkin-Elmer, Norwalk, CT) over the region 9 to 11 μm (Firestone and Sheppard, 1992). The standard was methyl elaidate at concentrations of 0 to 5 mg/mL.

RESULTS

Fatty Acid Composition of the Sauromatum Appendix

The amount of fatty acids in the appendix increased 2-fold during anthesis, from 2.7 mg/g fresh weight on D-3 to 6 mg/g fresh weight on D-2 (Table I). On D-day, 50% of total fatty acids was consumed. The major fatty acids present in the appendix tissue during development were palmitic, oleic, cis-vaccinic, linoleic, and linolenic acids (Table II). The levels of palmitic, stearic, linoleic, and linolenic acids fluctuated only slightly during development. The most noticeable change was the interchange in ratio between oleic and cis-vaccinic acids. During premature stages from D-19 until D-3, oleic acid accounted for more than 10% of the total fatty acids, whereas 6 cis-vaccinic acid was less than 5%. On D-3, the ratio of oleate to vaccinate

<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>mg/g fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-3</td>
<td>2.7 ± 0.0</td>
</tr>
<tr>
<td>D-2</td>
<td>6 ± 0.9</td>
</tr>
<tr>
<td>D-1</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>D-day evening</td>
<td>2.9 ± 1.5</td>
</tr>
</tbody>
</table>
Table II. Composition of C\textsubscript{16} and C\textsubscript{19} fatty acid series in the appendix of the S. guttatum inflorescence during development

One appendix was used for each stage of development: for example, the data for D−5 morning (around 9:00 AM) and evening (around 9:00 PM) were obtained from one appendix. The following fatty acids were present in amounts less than 2%: 12:0, 14:0, 18:0, 20:0, 20:1, 22:1, 22:1, 24:0. Values are expressed as percentages of total fatty acids (the average of two determinations).

<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>Total Fatty Acids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td>D−19 morning</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>D−14 morning</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>D−5 morning</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>D−5 evening</td>
<td>20 ± 0.1</td>
</tr>
<tr>
<td>D−3 morning</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>D−3 evening</td>
<td>20 ± 0.5</td>
</tr>
<tr>
<td>D−2 morning</td>
<td>20 ± 0.01</td>
</tr>
<tr>
<td>D−2 evening</td>
<td>19 ± 0.7</td>
</tr>
<tr>
<td>D−1 morning</td>
<td>20 ± 1.5</td>
</tr>
<tr>
<td>D−1 evening</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>D-day morning</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td>D-day evening</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>D+1 morning</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>D+2 morning</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>

changed from 3:1 in the morning to almost 1:1 in the evening. On D−2, the percentage of oleic acid was further reduced, whereas that of cis-vaccinic acid increased. Oleate reached a low level of 2% during heat production versus that of 12% at young stages. Vaccinate, on the other hand, reached a high level of 17% during thermogenic activity, although at young stages it accounted for only 0.8% of the total fatty acids. The percentage of palmitoleate [16:1 (9)], the common precursor of cis-vaccinate [18:1 (11)], remained low throughout the development of the appendix.

The absolute amounts of palmitoleic, oleic, and cis-vaccinic acids were 10 µg, 243 µg, and 81 µg/g fresh weight, respectively, on D−3 morning, and 120 µg, 360 µg, and 660 µg/g fresh weight, respectively, on D−2 morning. The amount of palmitoleic acid increased 12-fold, cis-vaccinic acid increased 8-fold, and oleic acid increased 1.5-fold.

The use of a more polar fused silica capillary column (DB-23) provided better resolution of the methyl esters of the 18:1 isomers; oleate and trans-vaccinate still could not be resolved, but cis-vaccinate and elaidate were isolated as single peaks (Fig. 2A). trans-Double bonds were not detected by FTIR in an extract prepared from D−2 and D−1 appendices, suggesting that trans-vaccinic acid was not present in the appendix tissue. Therefore, the second peak from the right in Figure 2, B and C, corresponds to oleic acid only. The chromatograms clearly show that the percentage of oleic acid declined, whereas that of cis-vaccinic acid increased. The mass spectra of the isomers were identical (data not shown) with those of the authentic samples and published spectra (Sun and Holman, 1968). The percentage of oleic acid was negatively correlated with that of cis-vaccinic acid during development (Fig. 3).

Fatty Acid Composition of Various Organs of the Sauromatum Inflorescence

The major constituents of fatty acids in the organs of the inflorescence were palmitic and linoleic acids, the latter of which was the most prevalent, accounting for about 40% of total fatty acids. Linoleic acid was detected by FTIR in an extract prepared from D−2 and D−1 appendices, suggesting that trans-vaccinic acid was not present in the appendix tissue. Therefore, the second peak from the right in Figure 2, B and C, corresponds to oleic acid only. The chromatograms clearly show that the percentage of oleic acid declined, whereas that of cis-vaccinic acid increased. The mass spectra of the isomers were identical (data not shown) with those of the authentic samples and published spectra (Sun and Holman, 1968). The percentage of oleic acid was negatively correlated with that of cis-vaccinic acid during development (Fig. 3).

Figure 2. Total ion current chromatograms of the 18:1 series of the Sauromatum appendix at two stages of development. A, A mixture of authentic fatty acid methyl esters of 18:1 isomers: elaidate acid (trans 9), oleate (cis 9), vaccinate (cis or trans 11). B, Fatty acid methyl esters from a D−2 appendix. C, Fatty acid methyl esters from a D−1 appendix. Each chromatogram was normalized to its largest peak.
gans along the spadix linoleic acids, whereas the levels of cis-vaccinic, palmitoleic, and vaccinic acids during development of the appendix. The percentage of cis-vaccinic acid versus oleic acid among total fatty acids versus the percentage of oleic acid was plotted. The value of the slope (−1.25) of the most fitted curve was compared to −1.0 using a t-test. The critical value for the slope is not significantly different from −1.0.

Table III: Composition of C₁₆ and C₁₈ fatty acid series in various organs of the Sauromatum inflorescence on D-day (the position of the organs along the spadix is shown in Fig. 1)

The spadix was divided into two regions: the lower, thermogenic region close to the female flowers, and the nonthermogenic region close to the male flowers, which are thermogenic as well. The following fatty acids were present in amounts less than 2%: 12:0, 14:0, 20:0, 20:1, 20:2, 22:0, 22:1, 22:2. Values are expressed as percentage of total fatty acids (the average of two measurements).

<table>
<thead>
<tr>
<th>Organ</th>
<th>16:0 ±</th>
<th>16:1 ±</th>
<th>18:0 ±</th>
<th>18:1 (9) ±</th>
<th>18:1 (11) ±</th>
<th>18:2 ±</th>
<th>18:3 ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peduncle</td>
<td>26 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
<td>1 ± 0.6</td>
<td>39 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Spathe</td>
<td>20 ± 0.2</td>
<td>2 ± 1</td>
<td>4 ± 0.2</td>
<td>3 ± 0.1</td>
<td>2 ± 0.07</td>
<td>49 ± 0.6</td>
<td>9 ± 0.02</td>
</tr>
<tr>
<td>Female flowers</td>
<td>25 ± 1</td>
<td>0.9 ± 0.03</td>
<td>2 ± 0</td>
<td>5 ± 0.3</td>
<td>1 ± 0.1</td>
<td>53 ± 1</td>
<td>9 ± 0.3</td>
</tr>
<tr>
<td>Lower spadix</td>
<td>25 ± 0.7</td>
<td>0.6 ± 0.2</td>
<td>1 ± 0.1</td>
<td>9 ± 0.2</td>
<td>0.7 ± 0.02</td>
<td>53 ± 4</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Club-shaped organs</td>
<td>26 ± 0.5</td>
<td>2 ± 0.1</td>
<td>1 ± 0.06</td>
<td>1 ± 0.2</td>
<td>6 ± 4</td>
<td>39 ± 0.4</td>
<td>25 ± 0.02</td>
</tr>
<tr>
<td>Low spadix</td>
<td>30 ± 0.6</td>
<td>0.3 ± 0.07</td>
<td>1.3 ± 0.02</td>
<td>2 ± 0.07</td>
<td>3 ± 0.6</td>
<td>31 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Male flowers</td>
<td>27 ± 0.9</td>
<td>0.4 ± 0.3</td>
<td>2 ± 0.2</td>
<td>3 ± 0.6</td>
<td>3 ± 0.2</td>
<td>23 ± 1</td>
<td>35 ± 1</td>
</tr>
</tbody>
</table>

5%, whereas in D. edule var angustifolium it changed very little, from 10 to 9%. During this same interval the level of linoleic acid dropped in sporophylls of E. ferox and D. edule var angustifolium but not in sporophylls of D. edule var edule. The level of linolenic acid fluctuated in the opposite direction from that of linoleic acid in those three species. These changes seemed to be species specific and they reflect the high metabolic activity of these organs.

**DISCUSSION**

Tissue-specific variations in the fatty acid composition of four thermogenic species occur during anthesis. In the thermogenic cycads, lipid respiration (Tang et al., 1987) and the production of volatiles derived from fatty acids during heat production (Pellmyr et al., 1991) may be the reasons for the changes in fatty acid composition. In the Sauromatum appendix, the inverse change in the levels of two 18:1 isomers, oleic and cis-vaccinic acids, may also reflect the production of volatile fatty acids (H. Skubatz, D.D. Kunkel, J.M. Patt, W.N. Howald, and B.J.D. Meuse, unpublished data) and/or the production of heat.

cis-Vaccinate occurs at high levels (more than 10% of total fatty acids) in some seed oils (Chisholm and Hopkins, 1965; Kleiman and Payne-Wahl, 1984), fruit pulp (Shibahara et al., 1986, 1987; Hibahara et al., 1989; Yamamoto et al., 1990), and tissue cultures of Hydnocarpus anthelmintica (Spencer et al., 1974) and Petroselium crispum (Ellenbracht et al., 1980). However, in most plants (Chisholm and Hopkins, 1965; Mukherjee and Kiewitt, 1980), as well as mammals (Holloway and Wabil, 1964; Kuemmler and Chapman, 1968; Longmuir, 1987) and microorganisms (Fulco, 1983), it is present in low concentrations (0.5–2%).

It is generally accepted that the oleic acid is almost exclusively derived via Δ⁹ desaturation of stearic acid, and that vaccinic acid is formed by Δ⁹ desaturation of palmitic to palmitoleic acid and subsequent C₂ elongation (Inkpen and Quackenbush, 1969). A new isomerase responsible for...
the conversion of oleic to cis-vaccinic acid and vice versa has been reported in pulp of several fruits (Hibahara et al., 1989; Shibahara et al., 1990; Shibahara, 1993). However, this isomerization reaction represents a minor pathway in those fruits. Rather, most of the cis-vaccinic acid is derived from palmitoleic acid.

In the *Sauromatum* appendix, palmitoleic acid, the common precursor of vaccinic acid, is a minor component during anthesis, and its absolute amount increases from 10 μg/g fresh weight on D−3 to 120 μg/g fresh weight on D−2. The amounts of cis-vaccinic acid and oleic acid are 660 μg/g fresh weight and 360 μg/g fresh weight, respectively, on D−2. Even though the level of palmitoleic acid is low, it can continuously be synthesized and converted to cis-vaccinic acid. The amount of linoleic acid that is synthesized from oleic acid increases from 1.1 mg/g fresh weight on D−3 to 3.3 mg/g fresh weight on D−2. This increase may contribute to the small increase observed in the amount of oleic acid. On the other hand, the inverse correlation between the percentages of oleic acid and cis-vaccinic acid may suggest that oleic acid is the precursor of cis-vaccinic acid. Since the amounts of individual fatty acids do not change stoichiometrically, the route(s) of the synthesis of cis-vaccinic acid in the appendix tissue is still uncertain.

It has been shown that cis-vaccinic acid is incorporated into glycerolipids (Kates et al., 1979; Ellenbrach et al., 1980) and possibly into specific molecular classes of phospholipids (Weber et al., 1979). This incorporation into the membrane lipids may alter the membrane structure and function. cis-Vaccinic acid has been found in all the thermogenic organs examined by us thus far, but its cellular distribution has not yet been studied. Therefore, its role, if any, in thermogenicity has to be elucidated. Since the *Sauromatum* appendix and the cycad cones emit fatty acid volatiles, on D−day 50% of total fatty acids, including cis-vaccinic acid may be involved in the emission of these volatiles. On D−day 50% of total fatty acids, including cis-vaccinic acid is consumed, and it may well be that the acid serves also as a precursor for various fatty acid-derived volatiles.

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