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Mechanisms for elongation in the biosynthesis of fatty acid components of epi-cuticular waxes

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Abstract

It is known that branched-chain amino acids can serve as precursors to iso- and anteiso-branched components of epi-cuticular waxes. Keto acid deamination products of Val, Leu and Ile are thought to serve as primers which are elongated by fatty acid synthase. However, the origin of elongation carbons has not been studied directly. Nor has the mechanism for formation of odd-carbon-length, straight- or branched-chain, cuticular ester fatty acids or free odd-carbon-length, straight fatty acid components of waxes been characterized. It is not known that α-oxidation of even-length precursors or elongation of odd-length primers is involved in these cases. Here, we present evidence which substantiates the expectation that elongation of branched as well as straight-chain precursors to wax ester acids occurs by fatty acid synthase catalyzed by addition of two carbon units via acetate. Also, we present evidence which indicates that odd-carbon-length acids can result from elongation of odd-carbon-length primers (at least branched), rather than even-length acids shortened by α-oxidation.

Keywords: *Brassica*; *Nicotiana*; Biosynthesis; Chain elongation; Epi-cuticular wax; Wax acids

1. Introduction

Epicuticular waxes (EW) that coat the outer, aerial surfaces of plants serve to protect the plant from desiccation, as a barrier to pests, and may function in leaf temperature control, frost hardiness, signaling between pollen and stigma, etc. (von Wettstein-Knowles, 1995; Lemieux, 1996). Main components of EW include *n*-alkanes, alkyl esters, *n*-alkanoic acids, primary and secondary alcohols, aldehydes, mono and diketones (Kolattukudy, 1980; Bianchi, 1995). Alkanes, free and esterified alcohols, and fatty acids (FA) having branched carbon backbones are common, but minor components of EW (Kolattukudy, 1980). Branched structures are usually iso- or anteiso in character. Alkanes, secondary alcohols and ketones having odd-carbon-length due to the loss of the terminal carbon in decarbonylation are common EW constituents.

Branched chain amino acids (Thr, Leu, Ile, Val) were shown to donate carbon to branched alkanes and FA in tobacco (Kolattukudy, 1968; Kaneda, 1967). It was suggested that after deamination of Leu, Val and Ile, resulting alpha keto acids might serve as primers for fatty acid synthase (FAS). But, the mechanism of elongation has not been verified. Also it is not known for certain if α-oxidation plays a role in formation of odd-carbon-length wax acids. Macey and Barber (1970) suggested that 15:0 of *Brassica oleracea* wax is formed by α-oxidation of 16:0 because the latter was reduced in a mutant while 15:0 was increased. Also, odd-length FA are formed in in vitro reactions from radiolabeled 16:0 or 18:0 (Cassagne & Lessire, 1978; Bognar, Paliyath, Rogers & Kolattukudy, 1984). And, Kolattukudy showed the appearance of small amounts of labeled 17:0 after feeding chopped tobacco leaves with radiolabeled 16:0 or 18:0 (Kolattukudy, 1966). However, the possible involvement of α-oxidation was not assessed directly, e.g. using approaches that moni-

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tor specific carbons of radiolabeled acids. One obstacle to studying this and other aspects of EW acid formation is the relative low level of incorporation of precursors into acid and ester–acid components of EW. The biochemistry and molecular biology of wax production in plants was recently reviewed (Post-Beittenmiller, 1996).

We have described efficient methods for analyzing radiolabeling patterns of FA (Kroumova & Wagner, 1995). Here we apply these methods to analysis of the metabolic origin of elongation carbons of odd- and even-length free and wax ester FA of EW derived from tobacco, kohlrabi and Brussels sprouts.

2. Materials and methods

2.1. Radiolabeling of Tissues

All tissue incubations were at 26°C with constant illumination by a tungsten lamp in a chamber that provided high humidity. Tissues were labeled for 20–48 h with $60–100\times10^6$ dpm of [1-^{14}C] Na acetate or [2-^{14}C] Na acetate (59 μCi/mmol, Moravek Biochemicals, Brea, CA). In all cases 90% or more of supplied label was taken up by tissues. After uptake, label solution was replaced with H_2O.

Three to four young leaves of *Nicotiana tabacum* cv. KY14 and cv petit havana were supplied label through the petiole for 24 h. For *N. glauca* and *N. tabacum* cv. NC 2326, lower leaf epidermis was peeled from young leaves and peels (about 20 cm^2) were floated on 3 ml, 0.75-strength Hoagland's solution containing label, and were incubated for 20 h.

Three to four young leaves of cabbage, broccoli (*Brassica oleracea* var. Red Danish) and Brussels sprouts (*B. oleracae* var. Catskill) plants were supplied label through the petiole for 48 h. Leaf discs (0.6 cm diameter) of Brussels sprouts were floated on labeling solution for 20 h.

2.2. Recovery of wax constituents and Schmidt degradation

For *N. tabacum* cv KY14, the procedure of Arrendale et al. (1988) was modified to recover wax components. After incubation with label, leaves or epidermis were washed 8 times (2 s each) with methylene chloride to remove EW. The solvent was evaporated, and the residue was partitioned with a mixture of hexane and 80% methanol (40:20 ml). The hexane fraction was washed twice with 5 ml 80% MeOH and the MeOH fraction was washed with hexane (2 times 5 ml). The hexane fraction and wash were combined and shaken with 10 ml saturated KCl (2 times), then dried with anhydrous Na_2SO_4. Solvent was evaporated and the residue containing hydrocarbons, esters, fatty alcohols and acids was saponified for 2 h at 80°C with 5 ml 2 N KOH in 80% MeOH. After cooling, FA were extracted using the procedure of Arrendale, Severson & Chortyk (1988). Free FA were dissolved in 85% acetonitrile, 0.01 M H_3PO_4 and then separated by HPLC (Kroumova & Wagner, 1995) using a gradient of 60–100% acetonitrile, 0.01 M H_3PO_4. In the cases of *N. tabacum* cv. petit havana, NC2326, and *N. glauca*, the above procedure was followed except that after saponification, chloroform extraction was used to remove hydrocarbons, alcohols, ketones, etc., but not potassium acid salts. The residue containing K salts was acidified with HCl and free acids were extracted with petroleum ether (b.p. 60–80°C). Solvent was evaporated and acids were separated by HPLC as above. For *Brassica* species, after incubation, leaves were dipped in $CHCl_3$ for 30 s, solvent was evaporated, and the residue was shaken with hexane:80% MeOH (40:20 ml). The hexane fraction was then processed as described above. For leaf discs of Brussels sprouts, methylene chloride was used as extractant instead of $CHCl_3$ to avoid extraction of chlorophyll from cut surfaces. Schmidt degradations were made as previously described (Kroumova & Wagner, 1995).

3. Results and discussion

Radiolabeling of total EW components of both *Nicotiana* and *Brassica* plants tested was between 0.7 and 11.6% of [2-^{14}C] Na acetate taken up (Table 1). This compares with e.g. typical results of Kolattukudy (1965) where incorporation of this precursor into waxes was 2.5–7%. After saponification, the bulk of label was extracted into $CHCl_3$. This material presumably consists of alkanes, alcohols, ketones and other non-saponifiable components (Table 1). Total free and esterified fatty acid (FA) constituted about 1–8% of EW label. Therefore, percent of total uptake into free plus esterified (wax ester) FA was 0.01–0.46%. Despite this low incorporation (typical of EW labeling), sufficient labeling of EW FA was achieved to allow recovery of separated acids (HPLC of free acids). As shown in Table 2, depending upon the plant, labeled 14:0–22:0 FA were recovered. But, the bulk of the label was presumably in longer chain (>C22) FA not separated by the C18 HPLC system used.

Perhaps the most detailed chemical characterization of free and esterified EW FA is found in the work of

Table 1
Incorporation of [2-^{14}C] acetate in epicuticular wax components of different plants

	Uptake (dpm×10^6)	Label in EW (dpm ×10^6 (% of uptake))	Label in alkanes, alcohols, ketons, etc. non-saponifiable components (dpm×10^6 (% of EW))	Label in total FA-free and esterified (dpm×10^6 (% of EW))	% of total uptake in free and esterified FA (%)
Nicotiana					
KY14 leaves	59.2	0.42 (0.7)	–	0.02 (4.7)	0.03
Petit havana leaves	91.1	1.44 (1.6)	1.27 (88.0)	0.02 (2.8)	0.05
N. glauca leaf epidermis	54.0	3.56 (6.7)	2.44 (6.5)	0.05 (1.3)	0.09
NC2326 leaf epidermis	70.5	0.73 (1.04)	–	0.056 (7.6)	0.08
Brassica					
Kohlrabi leaves	84.7	9.8 (11.6)	9.2 (93.9)	0.04 (0.4)	0.46
Cabbage leaves	27.1	0.64 (2.36)	0.57 (89.3)	0.009 (1.5)	0.04
Broccoli leaves	14.45	0.8 (5.5)	0.77 (96.0)	0.01 (1.2)	0.07
Brussels sprouts leaves	97.9	0.7 (0.7)	0.67 (94.3)	0.012 (1.6)	0.01
Brussels sprouts leaf discs	19.0	0.19 (1.0)	0.17 (91.0)	0.0034 (1.8)	0.02
Cabbage leavesa[a]		(2.5–7)			

[a] Data of Kolattukudy (1965).

Arrendale et al. for *N. tabacum*, c.v. NC2326 (Arrendale et al., 1988). The most abundant EW FA found in that study were those most prominently radiolabeled in the present study; 14:0, 15:0, 16:0, 18:0, 20:0 iso 15:0, anteiso 15:0. Similarly, for *Brassica* species, most prominently labeled FA found here were those found to be prominent in these species from chemical characterization studies (Macey & Barber, 1970; Baker & Holloway, 1975; Holloway, Brown, Baker, & Macey, 1977). In the cases of petit havana, cabbage (Table 2), and KY14 (not shown), no substantial label was found in the FA monitored here. Very long chain FA were not separated using the HPLC system applied and therefore these were not examined.

Table 2
Distribution of label in EW-derived fatty acids of various tissues[a]

FA	Tobacco (dpm and (% of total))			*Brassica* (dpm and (% of total))					
	N. glauca leaf epidermis	NC2326 leaf epidermis		petit havana leaves	broccoli leaves	Brussels sprouts discs	Brussels sprouts leaves	kohlrabi leaves	cabbage leaves
		exp. 1	exp. 2						
14:0	46 (0.1)	562 (1.0)	1148 (0.6)	–	354 (3.5)	123 (3.6)	177 (1.5)	620 (1.5)	–
15:0	–	270 (0.5)	268 (0.1)	–	146 (1.5)	43 (1.3)	100 (0.8)	302 (0.7)	–
i15:0	–	720 (1.3)	1150 (0.6)	–	–	74 (2.2)	–	–	–
a15:0[b]	–	630 (1.1)	368 (0.1)	–	–	–	–	–	–
16:0	516 (1.0)	800 (1.4)	1870 (1.0)	94 (0.5)	186 (1.9)	65 (1.9)	104 (0.9)	620 (1.5)	–
a17:0[b]	–	–	290 (0.15)	–	–	50 (1.5)	75 (0.6)	–	–
18:0	380 (0.8)	704 (1.3)	1150 (0.5)	45 (0.2)	–	52 (1.5)	86 (0.9)	–	–
i18:0[c]	–	–	–	–	–	59 (1.7)	86 (0.7)	–	–
a19:0[b]	–	–	–	–	–	–	–	147 (1.2)	–
20:0	672 (1.3)	1042 (1.9)	2140 (1.1)	–	–	–	–	169 (1.4)	–
22:0	1272 (2.5)	2566 (4.6)	–	–	–	–	–	–	–
Total label in FA	50,000	56,000	190,400	20,000	10,000	3,400	12,000	40,000	9,000

[a] FA >C-22 were not recovered.[b]Anteiso-branched.[c]Iso-branched.

Presumably only longer chain (>C22) free or esterified EW FA are produced in the tissues of these plants as labeled here.

Certain purified EW FA from NC2326, kohlrabi and Brussels sprouts leaves were selected for further analysis based on the amount of labeled acid recovered from HPLC, and on our interest in analyzing iso- and anteiso-branched as well as straight-chain acids. The acids 14:0, 15:0, 16:0, 18:0, iso-15:0, anteiso-15:0 and anteiso-17:0 were examined by Schmidt degradation to assess the pathway of chain elongation involved in their synthesis. It has been assumed in the literature that straight chain, free and esterified EW FA are formed from Malonyl CoA, elongated by addition of 2 carbon units (from acetate) via FAS. But, the possibility that longer (>2 carbons) straight chain primers might be utilized by FAS in EW FA formation has been little explored (Arrendale et al., 1988). For example, elongation of propionyl CoA by FAS could yield odd length, linear FA. It has also been assumed that odd length, straight-chain components are derived by shortening of even length precursors via α-oxidation (removal of carboxyl carbon). Also, it has been assumed that in the cases of iso- and anteiso-branched EW FA, primers are elongated by FAS to yield odd or even length, branched components. However, the mechanism of elongation in branched EW FA formation has not been studied directly.

There are two known possibilities for elongation to yield medium chain FA, FAS (2 carbon/elongation step) and α-KAE (one carbon/elongation step). The latter mechanism is only known to occur in glandular trichomes of tobacco and petunia (Kroumova, Xie, & Wagner, 1994), but not in glandular trichomes of the primitive tomato, *L. pinnellii* (Van der Hoeven & Steffens, 1997, and our unpublished observations) or *Datura inoxia* (unpublished). Since α-KAE can yield straight as well as branched medium chain FA, it may (however unlikely) be responsible for medium chain (up to C16) and perhaps long chain (>C16) FA of EW. This possibility is not unreasonable if one considers that tobacco trichome glands (where α-KAE occurs) and epidermis (where EW is formed) are of the same epidermal origin.

To distinguish between FAS and α-KAE elongation, one can compare labeling patterns in the carboxyl carbon of FA using Schmidt degradation (Kroumova et al., 1994; Kroumova & Wagner, 1995). If [2-^{14}C] acetate is the source of label, and no label randomization occurs, FAS will produce FA containing no label in the carboxyl carbon. In contrast, α-KAE will yield carboxyl-labeled FA (Kroumova et al., 1994). As shown in Table 3, carboxyl labeling in all acids tested from the 3 plants studied was low, within or close to the error of the method (~3%). Somewhat higher carboxyl label in [2-^{14}C] acetate labeled 14:0 and 15:0 of NC2326 and 15:0 of kohlrabi are probably due to the low level of these radiolabeled acids recovered from HPLC. Further, labeling with [1-^{14}C] acetate resulted in substantial carboxyl labeling. If α-KAE were involved, no carboxyl labeling would be expected. Results with 16:0 (normal product of FAS) may serve as an internal control in NC2326 and kohlrabi. Lack of carboxyl labeling in these cases indicates the lack of label randomization and argues against artifact due to randomization. It is noteworthy that Kolattukudy also found little evidence of randomization from [2-^{14}C] acetate in labeling EW of *Brassica* for up to 48 h (Kolattukudy, 1965).

The cases of i-15:0 and a-15:0 from NC2326 and a-17:0 from Brussels sprouts provide evidence that α-oxidation of elongated branched precursors is not involved in formation of odd-length, branched chain EW FA. If e.g. i-15:0 were derived from α-oxidation

Table 3
Radioactivity in carboxyl carbons of epicuticular wax FA, labeled with [2-^{14}C] or [1-^{14}C] acetate (% of total)

FA	NC2326 [2-^{14}C }] acetate	Kohlrabi		Brussels sprouts	Expected radioactivity	
		[1-^{14}C] acetate	[2-^{14}C] acetate	[2-^{14}C] acetate	[1-^{14}C] acetate	[2-^{14}C] acetate
14:0	5.0	14.1, 11.7	1.9, 2.6	l	14.3	0
15:0	4.3	8.9, 8.3	6.3	l	11.6 to 16.6 or 0[a]	0 or 12.5[a]
16:0	2.6, 1.2	12.3, 17.2	2.2, 5.8	l	12.5	0
18:0	1.2	n.e.	n.d.	l	11.1	0
i15:0	2.3	n.l.	n.l.	n.l.		0 or 11.1 to 16.6[a]
a15:0	3.3	n.f.	n.f.	l		0
a17:0	n.f.	n.l.	n.l.	1.8		0

n.f. = not formed; n.e. = not examined; n.l. = not labeled; l = low label[a]The first value or range is that expected without α-oxidation, and the second is that with α-oxidation involved. Ranges consider variation due to extent of label in primers that are elongated to form these fatty acids.

of FAS-formed i-16:0, then its carboxyl carbon should contain 12.5% of total acid label. Yet only a low (background) level was found. Similar results were found for a 15:0 of NC2326 and a-17:0 of Brussels sprouts. This suggests that odd-length primers are elongated by FAS to form these components. The putative primers for i-15:0, and both a-15:0 and a-17:0 are leucine-derived, 3-mebutyryl CoA, and isoleucine-derived 2-mebutyryl CoA, respectively.

Similarly, results obtained for 15:0 of NC2326 and kohlrabi also suggest the lack of participation of α-oxidation in formation of this odd, straight-chain component (Table 3). Low (4.3% and 6.3%) label was in the carboxyl carbon of these, respectively, while 12.5% was expected if α-oxidation were involved. Carboxyl labeling in 15:0 from [1-^{14}C] acetate labeled kohlrabi was lower than that expected if α-oxidation of 16:0 were not involved. Only about 8.6% carboxyl labeling was found while 11.1–16.6% was expected (range depending on labeling in the primer). The reason for this somewhat low carboxyl labeling in this case is not clear. Label randomization is not evident given the similarity of observed and expected carboxyl labeling in 14:0 and 16:0 of kohlrabi labeled with either [1-^{14}C]- or [2-^{14}C] acetate (Table 3).

With the possible exception of results for [1-^{14}C] acetate labeled 15:0 of kohlrabi, Table 3 data suggest the lack of participation of α-oxidation in the formation of branched or straight-chain EW FA components examined in this study, using 3 different plants. It is noteworthy that we recently obtained evidence that pentanoyl, heptanoyl and nanonyl groups of *Datura metel* sugar esters are formed from odd-length precursors elongated by FAS, without participation of α-oxidation (unpublished results).

Results presented here are consistent with the expectation that EW FA are formed by FAS elongation beginning with malonyl CoA or utilizing primers (including propionyl CoA, toward straight-chain, odd carbon length products) derived from branched-chain amino acid metabolites.

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