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WAX SYNTHESIS IN *BRASSICA OLERACEA* AS MODIFIED BY TRICHLOROACETIC ACID AND GLOSSY MUTATIONS

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Abstract—Two different mutations in *Brassica oleracea*, gl_5 and gl_4, have been re-investigated using acetate-1-^{14}C labelling in an attempt to define more closely the nature of the genetic blocks to wax synthesis. It has been found that gl_5 is a mutation which blocks elongation in the step C_{28}–C_{30} The mutation gl_4 exhibits no elongation block and could be blocked in the decarboxylation step C_{30}–C_{29} 0·1 mM TCA supplied in the culture solution of cauliflower seedlings affected the leaf surface by producing a glossy appearance similar to that induced by gl_3 and gl_4 At this concentration growth was not inhibited and the appearance of the plants was normal except for the surface wax The amount of surface wax produced was about 40% of that in untreated seedlings on a leaf area basis Slight, but significant changes in wax composition were noted, mainly involving a reduction in C_{30} acids and aldehydes, a slight reduction (33–29%) in alkane content, and a marked difference in chain length composition of the alkanes with C_{27} increased relative to C_{29} Over a range of concentrations from 0·1–1 mM, TCA inhibited incorporation of label from acetate-1-^{14}C into C_{30} acids and aldehydes more than into C_{28} at concentrations 0·4–0·8 mM while label tended to accumulate in C_{24} and C_{26} acids, thus elongation C_{28}–C_{30} was especially sensitive to TCA TCA also inhibited incorporation into primary alcohols and esters almost as much as into C_{29} compounds In spite of relatively specific effects on incorporation of label into longer chain lengths, the resulting block to C_{30} synthesis is not sufficient to make much difference to the overall rate of C_{29} synthesis Both results of analysis of wax from whole plants and experiments with tissue slices *in vitro* indicated that the effect of TCA in reducing the glaucousness of the leaf surface is a combination of overall reduction of wax synthesis together with slight but significant changes in wax composition

INTRODUCTION

CHEMICAL analysis of glossy mutants in *Brassica* and *Pissum*[1,2] has indicated that some of these, for example gl_5 in *B. oleracea*, show evidence of a block in the elongation of very long chain fatty acids from C_{24}–C_{30}. The mutant gl_5 showed much reduction of *n*-C_{30} and C_{28} in the free acid of the wax, suggesting a partial genetic block in the step C_{26}–C_{28}, or in some cases C_{28}–C_{30}. The position of the block could not be determined by chemical analysis only. Other glossy mutations, such as gl_4 in *Brassica*, did not show any inhibition of the formation of the C_{30} acid and on the contrary showed a tendency to accumulate this component. In both mutants, the main phenotypic effect was a reduction in nonacosane from 33% of the wax to 2–6%. Together with this the total amount of wax was reduced to about the same extent as the reduction in C_{29} paraffins, ketones and secondary alcohols, and the resulting wax crystallites were completely modified in physical character[3] or eliminated, thus producing the glossy surface.

[1] MACEY, M J K and BARBER, H N (1970) *Phytochemistry* **9**, 13
[2] MACEY, M J K and BARBER, H N (1970) *Phytochemistry* **9**, 5
[3] HALL, D M, MATUS, A I, LAMBERTON, J A and BARBER, H N (1965) *Aust J Biol. Sci* **18**, 323

Kolattukudy[4] has adduced much evidence in favour of elongation-decarboxylation as a mechanism for the formation of C_{29} paraffin in *Brassica*, although long chain fatty acid condensation has in the past been favoured [5,6] As stated elsewhere[7] most of our own results can now be interpreted in terms of Kolattukudy's hypothesis. There is now some evidence that secondary alcohol and ketone might be directly derived from alkane [8]

Recently, thiol inhibition of alkane synthesis in peas has been reported.[9] This inhibition appears to mimic gl_4 action in that C_{32} compounds accumulate, and it was assumed that the decarboxylation step was inhibited by the very high (10 mM) conc of dithiothreitol used It has also been reported in unpublished work recently cited[10] that inhibition of wax synthesis by TCA is more selective on longer chain lengths These effects of TCA have now been investigated, with particular attention to comparisons of inhibitor and mutant effects on wax synthesis.

TABLE 1 INCORPORATION OF ACETATE-1-^{14}C BY TISSUE SLICES OF *Brassica oleracea* MUTANTS gl_5, gl_4 AND NORMAL PLANTS

Wax component	gl_5	Normal cauliflower	gl_4	Normal kale
Free acid	1 017	0 640	0 476	0 456
Primary alcohol	0 399	0 319	0 265	0 499
Secondary alcohol	0 010	0 181	–	0 218
Aldehyde	1 830	1 464	1 668	2 271
Ketone	0·069	0 466	0 068	0 385
Ester	0 351	0 321	0 143	0 239
Paraffin	0 008	1 527	0 032	1 747
% Acetate → wax conversion	3 38%	4 42%	2 38%	5 23%

Tissue slices were prepared from 2 g leaf tissue and incubated for 4 hr in 4 ml 0 05 M phosphate buffer pH 7 2 containing 50 μmol $KHCO_3$ and 5 μC acetate-1-^{14}C (sp act 55 mC/m mol) Temperature in the flasks was 31 5° ± 0 5° Results are expressed in dpm × 10^5 quenching corrections being by the Channels Ratio Method

RESULTS AND DISCUSSION

Table 1 shows acetate-1-^{14}C incorporation into the wax of two leaf mutants, gl_5 and gl_4 and into their corresponding normal forms The material for the experiment was obtained from a segregating F_2 generation and both normal forms are included for comparison with the corresponding mutant. Both mutants caused a large reduction in incorporation of label into alkanes, ketones and secondary alcohols The gl_5 mutation caused accumulation of label in the free acids, but otherwise the overall labelling pattern was quite similar for the two mutants

[4] KOLATTUKUDY, P E (1970) *Ann. Rev Plant Physiol* **21**, 163
[5] CHANNON, H J and CHIBNALL, A C (1929) *Biochem J* **23**, 168
[6] MACEY, M J K and BARBER, H N (1969) *Nature* **222**, 789
[7] MACEY M J K (1970) *Phytochemistry* **9**, 757
[8] KOLATTUKUDY, P E and LIU TSUI-YUAN J (1970) *Biochem Biophys Res Commun* **41**, 1369
[9] BUCKNER, J S and KOLATTUKUDY, P E (1973) *Arch Biochem Biophys* **156**, 34
[10] KOLATTUKUDY, P E and WALTON T J (1973) *Progress in the Chemistry of Fats and Other Lipids* by (HOLMAN, R T ed), Vol XIII, Part 3 Pergamon Press Oxford

Table 2 analyses the label distribution in various components of acids and aldehydes of gl_5, gl_4 and one normal form. In gl_5, the genetic block must be between C_{28} and C_{30}, because although C_{28} acid labelling is reduced relative to C_{26} acid, the C_{28} acid is appearing as aldehyde, probably as a result of accumulation of the acid before the block. The mutant gl_4 does not show this feature, but the labelling of the C_{30} component shows only a slight accumulation of label compared with the normal form. Previous work[1] has shown that in mature leaves, in which the effect of the mutation has been integrated for a long growing period, the percentage composition of aldehydes was : Normal, C_{28} 49·3%, C_{30} 36·1% with smaller amounts of other components; gl_4, C_{28} 7·7%, C_{30} 75·4% with smaller amounts of other components. In the free acids the effect of the genetic block was not so apparent, presumably because accumulation of the C_{30} acyl group promotes conversion to the aldehyde in the absence of a pathway for decarboxylation.

TABLE 2 DISTRIBUTION OF RADIOACTIVITY (dpm × 10^3) IN EACH PROMINENT COMPONENT OF ALDEHYDES AND FREE ACIDS OF NORMAL AND MUTANT FORMS OF *Brassica oleracea* FOLLOWING INCUBATION OF SLICED TISSUE WITH ACETATE-1-^{14}C

Carbon no	Acids			Aldehydes		
	gl_4	Normal	gl_5	gl_4	Normal	gl_5
C_{24}	8 61	9·21	37 7	—	—	—
C_{26}	13·9	21 8	51 6	—	—	—
C_{28}	9 6	11 1	12 3	21 5	21 3	100 4
C_{30}	15 3	21 8	—	145 3	124·3	8 8

The material was isolated from the experiment giving the results of Table 1, using the normal cauliflower for comparison with mutants

Buckner and Kolattukudy[9] have shown that thiols such as dithiothreitol and mercaptoethanol caused accumulation of C_{32} aldehydes in peas with concomitant inhibition of C_{31} alkane synthesis. They observed also that TCA specifically (0·625 mM) inhibited the incorporation of label into C_{32} aldehyde but not into C_{28} or C_{26} aldehydes. These results very much resemble those reported above. TCA effects appear to mimic the action of the gl_5 mutation (but only partially as shown below) and thiols the gl_4 mutation. Similar mutations exist in *Pisum*,[2] where *wa* and *wb* are apparently elongation mutations and *wsp* a decarboxylation mutation. From the results of chemical analysis, it can be postulated that mutation *wb* affects the C_{26}–C_{28} step, *wa* apparently C_{28}–C_{30}, and *wsp*, presumably affecting decarboxylation, accumulates C_{32} aldehyde and acid. The manner of genetic control of elongation in such a specific manner is a matter of some importance, because the implication is that different enzymes could be involved in different but analogous steps in the elongation sequence.

Work with inhibitors persuaded Buckner and Kolattukudy[9] that the C_{32} aldehyde was closely related to the C_{31} alkane in peas. The above shows that the C_{30} aldehyde of *B. oleracea* is affected by the same mutation as the free acid and the C_{29} alkane, so that the situation in *B. oleracea* is analogous to that in *Pisum*. Buckner and Kolattukudy[9] state that the C_{26} and C_{28} aldehydes are related to the primary alcohols, rather than to the alkanes. This is borne out by previous work on pea wax,[2] where the mass distributions of C_{26} and C_{28} primary alcohols follows closely that for corresponding aldehydes. The situation in *B. oleracea* is different in that the primary alcohol and esterified alcohols and

acids contain branched chain components. The mass distribution of the normal carbon chains does not correspond well between alcohols and aldehydes. The reason for this difference between *B. oleracea* and *P sativum* is not clear, the latter does not contain branched chain components in the wax

Table 3 Chain lengths of various wax components as affected by TCA (10^{-4} M) in solution culture of *Brassica oleracea*

Chain length	Paraffins		Aldehyde		Acid*	
	Normal	TCA	Normal	TCA	Normal	TCA
C_{24}				-		
C_{25}	-					-
C_{26}					10·2	18·6
C_{27}	0·1	5·7				
C_{28}	-	-	23·1	53·9	30·3	56·4
C_{29}	92·3	89·8	-		10·1	7·2
C_{30}		-	76·9	46·1	37·0	7·7
C_{31}	7·6	4·5		-		

* The free acid contained 6·7% C_{14}, 2·3% C_{15}, 1·7% C_{16} and C_{17} in normal plants TCA treated plants were not noticeably different in this range

Figures refer to mass per cent composition of paraffins, aldehydes or free acids of the wax calculated for chain lengths C_{24}–C_{31} only

The effect of TCA on wax synthesis

Whole plants were grown in solution culture with and without TCA 10^{-4} M. At this concentration TCA is quite specific for its effect on the wax surface, which is converted to a glossy one visually similar to that of the mutants gl_5 or gl_4. At higher concentrations growth malformations and inhibitions occur The total wax yield per unit area of surface from treated plants was 0·27 mg cm^{-2} and from corresponding normal ones, 0·67 mg cm^{-2} The effect of TCA on distribution of alkane chain lengths is apparent from Table 3 The change is small, but reproducible, TCA induces a slight but perceptible change in the content of C_{29} in comparison with C_{27} which becomes relatively much greater in the treated plants This effect is quite similar to that of both gl_3 and gl_4,[1] although less marked quantitatively. Examination of the free acid fraction showed that the chain length distribution was altered using TCA The main change concerned the C_{30} acid, which was much reduced relative to C_{26} and C_{28}, the other two main components (Table 3) Aldehydes were similarly, but less affected.

The gross composition of the wax extracted from glossy-leaved TCA-treated plants was investigated, but not much difference was found from normal plants The total alkane content varied from 33% in normal wax to 29% wax from TCA-treated plants Thus TCA at 0·1 mM inhibits the synthesis of all wax components over an extended period of uptake from culture solution Preliminary tests showed that 1 mM TCA was completely inhibitory to growth. Since uptake and transpiration would be expected to accumulate the compound, some detoxification is probably involved in uptake from the 0·1 mM solution so that the internal conc would be intermediate between 0·1 and 1 mM.

The effect of TCA on incorporation of acetate-1-^{14}C into wax was extensively investigated; the tissue was sliced and washed extensively with buffer solution and then shaken with buffer containing the appropriate concentration of TCA for 1 hr before introducing the radioactive substrate Subsequent analysis of the effect of TCA concentration on wax

labelling was found to correlate to some extent with chain length and this is summarized in Table 4. The components of C_{30} chain length are obviously the most affected by intermediate concentrations of TCA whilst the C_{26} and C_{24} fatty acids show a clear tendency to accumulate. There is a sharp cut-off in chain length selectivity between 0·8 and 1 mM. In spite of the evidence of increasing TCA action with increasing chain length of the elongated product, there is no evidence of marked selectivity as between C_{29} compounds and primary alcohols and esters as has been described for other work in peas.[9] In these experiments with chopped tissue the trends for all wax components, apart from the exceptions noted, were similar. TCA appears to reduce total extractable wax by 60% at the same time producing only a slight but definite and reproducible change in wax composition. The specific effect of TCA in inducing a glossy surface on the leaves is brought about by a reduction in total wax synthesized or a change in wax composition or perhaps both.

TABLE 4 THE EFFECT OF TCA ON LABELLING OF WAX COMPONENTS BY ACETATE-1-^{14}C EXPRESSED AS % OF CONTROL WITHOUT TCA

Wax components	Trichloroacetic acid (mM) 0·4	0·6	0·8	1·0
C_{29} + C_{30} acid + C_{30} aldehyde	91	83	63	24
C_{30} acid + C_{30} aldehyde	100	72	59	19
C_{28} acid + C_{28} aldehyde	104	91	80	35
C_{26} acid	106	115	130	62
C_{24} acid	95	110	101	55
Esters	90	102	74	49
Primary alcohols	87	78	68	36

2 g tissue was exposed to 10 μC acetate-1-^{14}C for 3·5 hr Total cpm in controls—C_{29} + C_{30} acid + C_{30} aldehyde, $6\,2 \times 10^5$, C_{30} acid + C_{30} aldehyde, $1\,28 \times 10^5$, C_{28} acid + aldehyde, $1\,01 \times 10^5$, C_{26} acid, $3\,25 \times 10^4$, C_{24} acid, $2\,4 \times 10^4$, esters, $9\,9 \times 10^4$, primary alcohol, $1\,61 \times 10^5$

A direct comparison between TCA effects and those of gl_5 in cauliflowers and gl_3 in kale shows that both appear to strongly affect a process somewhere in the elongation step C_{28}–C_{30}. Obviously the action of TCA is much less selective and has quite different results from that of the mutant, but the end result is phenotypically similar. Free acids accumulate in experiments with the gl_5 and with TCA-treated leaf tissue.

Previous work[11] on TCA dealt with whole leaves and showed very specific effects on C_{29} compounds; this kind of experiment produces very variable results, it being difficult to ensure consistent uptake of the substrate by petioles of leaves immersed in solutions. Chopped tissue, however, is hardly an ideal biological system and it could interfere with incorporation patterns in an unpredictable way However, this cannot be the whole reason, because complete selectivity of TCA (625 mM) has been claimed for the alkanes and C_{32} aldehyde of peas using chopped leaves.[9] Both growth of the plant *in vivo* using TCA at concentrations which do not inhibit growth, and experiments *in vitro* using acetate-^{14}C

[11] KOLATTUKUDY, P E (1965) *Biochemistry* **4**, 1844

labelling, lead to the conclusion that TCA affects later elongation steps somewhat selectively, without causing much change in the final wax composition The C_{30} acyl group presumably reaches a low level in the TCA-treated tissue which is just enough to slightly slow down C_{29} biosynthesis relative to other components. In this respect the action of gl_5 is much more specific than TCA (see Table 1). This mutation does not affect primary alcohol, ester, or aldehyde synthesis, and causes accumulation of labelled free acids in *in vitro* experiments. Although the main aldehyde of normal *B oleracea* can be C_{28} or C_{30}, inhibition of elongation in gl_5 causes aldehyde to accumulate mainly in the C_{28} component

EXPERIMENTAL

For culture soln growth studies, the sodium salt of TCA was added to Hoagland's soln as a conc 10^{-4} M The plants grown with TCA in the culture soln showed no growth abnormalities and grew at the same rate as untreated plants The effect on the waxy surface was specific In soln culture, *Brassica* could not tolerate a conc 10^{-3} M Wax was extracted when the plants were at the 6-leaf state Most other techniques were as previously described [7] TCA was added as the sodium salt to *in vitro* incubations of tissue slices Tissue slices treated with TCA were given pre-treatment of 60 min before adding substrate Temperature was controlled by a H_2O bath at 35° Temperature in the flasks was monitored and did not vary by >0 5 0 02 M bicarbonate was included in the buffer solution used for incubation, since it was found that this procedure did maximise elongation, and without it, differences could be partly obscured In the tissue slice method some differences in incorporation patterns occur from using whole leaves with petioles dipped in substrate With chopped leaves more aldehyde and more acid is formed than in whole leaves In results of a typical experiment acetate-1-^{14}C was incorporated into the various major wax components with the following percentage distributions, using leaves of the same age from the same plant — *Whole leaves* acid, 0 7%, primary alcohol, 4 1%, secondary alcohol, 10 4%, aldehyde, 7 9%, ketone, 22 4%, ester, 6 2%, paraffin, 47 5% *Tissue slices* acid, 4 1%, primary alcohol 8 0%, secondary alcohol, 7 4%, aldehyde, 19 9%, ketone 16 9%, ester, 9 2%, paraffin, 35 7% There is a bias in favour of labelled acids and aldehydes in tissue slices However the incorporation patterns within these components were not changed, and the efficiency of acetate-1-^{14}C incorporation is much greater using tissue slices

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