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Short Communication

Cutin Biosynthesis in *Vicia faba* Leaves

EFFECT OF AGE[1]

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Plant cuticle consists of a polymer of hydroxy fatty acids called cutin, which is embedded in wax. Although in recent years the chemistry and biosynthesis of the wax components have been studied to a certain extent (1, 6), cutin biosynthesis remains merely speculative. Some information on the structure of hydroxy fatty acids that make up the cuticle is available (2, 3). Recently a tracer method coupled to a novel method of cleavage of the polymer was developed to study the biosynthesis of this polymer (7). These techniques can be used to determine at what stage of leaf growth cutin biosynthesis occurs. The results described in this communication clearly demonstrate that the most rapidly expanding leaves of *Vicia faba* synthesize cutin most rapidly, and that cutin synthesis virtually stops when the leaf reaches full expansion.

V. faba plants grown at 22 C with a 16-hr day in a soil-sand mixture (2:1) were used in all experiments. Three-week-old plants with five or six leaves were selected in such a way that all top leaves were of about equal age, and the lower leaves of the different plants had comparable ages. Leaves of different age groups were collected and 12-mm diameter discs were cut from each. After the discs were washed thoroughly with water, 20 discs of each age group were placed at the bottom of 125-ml Erlenmeyer flasks with 0.5 ml of palmitic acid-1-^{14}C (55 mc/mmole) solution containing 7.5 $\times$ 10^6 cpm (70% efficiency) and prepared as described before (5). The discs were incubated in the radioactive solution for 3 hr at 30 C in a gyratory water bath shaker.

At the end of the incubation period the leaf discs were washed twice, each time with 50 ml of distilled water. The discs were then homogenized (Ten-Broeck) in water and centrifuged at 15,000*g* for 15 min. The residue was treated with 25 ml of a 2:1 mixture of chloroform and methanol for 15 min and then centrifuged at 15,000*g* for 15 min. This treatment was repeated four more times and the final residue was similarly treated twice with 25 ml each of tetrahydrofuran. Little ^{14}C was released into the solvent at this stage. The radioactivity remaining in the residue has been demonstrated to be primarily in cutin (7). This insoluble material was refluxed with $LiAlH_4$ in tetrahydrofuran (previously treated with solid KOH and then distilled over $LiAlH_4$) for 24 hr. The excess $LiAlH_4$ was decomposed by a careful addition of the reaction mixture into water. Dilute HCl was added and the lipids were extracted five times with ethyl ether, and the ether was evaporated under reduced pressure. The residue was dissolved in 0.3 ml of a 2:1 mixture of chloroform and methanol and 10 μl were taken for counting while 50 μl were applied to 0.5-mm thick Silica Gel G layers with a hydrogenolysate of unlabeled pure *V. faba* cutin as a standard (7). The chromatograms were developed with ethyl ether:hexane:methanol (8:2:1 v/v) as the solvent system in a lined tank. Dichromate-sulfuric acid spray and subsequent heating at 160 C or a spray 2′,7′-dichlorofluorescein was used to detect the components on the thin layer plates.

Palmitic acid-1-^{14}C was found to be the best precursor of cutin thus far examined with *V. faba*; about one-fifth of the administered acid was incorporated into the cutin (7). Besides, the major part of this label was contained in the dihydroxypalmitic acid fraction of the cutin. Therefore, palmitic acid appeared to be the most suitable substrate for measuring cutin synthesis. In Table I the amounts of radioactivity incorporated into the ether-soluble lipids produced by $LiAlH_4$ treatment of the insoluble material obtained from *V. faba* leaves of various ages are shown. The youngest leaves used in experiment 1 were younger than those used in experiment 2. Thus, the second leaves used in experiment 1 and the youngest leaves used in experiment 2 represented the most rapidly expanding stage of growth of *V. faba* leaves, and at this stage they incorporated palmitic acid most rapidly into cutin. As the leaves expanded, the incorporation rate dropped rapidly. The fully mature leaves incorporated very little palmitic acid into cutin.

Table I. *Incorporation of Palmitic Acid-1-^{14}C into Cutin of V. faba Leaves of Different Ages*

In experiment 1 20 discs, 12 mm in diameter were incubated at 30 C for 2 hr with 0.5 ml 8.7 $\times$ 10^6 cpm (70% efficiency) of palmitic acid-1-^{14}C (55 mc/mmole). In experiment 2, 10 discs (12 mm) were incubated at 30 C for 3 hr with 4.2 $\times$ 10^6 cpm of palmitic acid-1-^{14}C (55 mc/mmole). The incorporation into the cutin was measured as described in the text. Leaf 3 of experiment 1 was about fully mature, and leaf 2 of experiment 2 was nearly mature. The two experimental results are presented in a staggered manner to denote the difference in the ages of the leaves used in the two experiments.

Position of the Leaf (from top)		Incorporation of Palmitic Acid-1-^{14}C	
Expt 1	Expt 2	Expt 1	Expt 2
		%	%
1 (youngest)		11.2	
	1		14.7
2		16.3	
	2		3.3
3		1.7	
	3		1.0
4		1.5	
	4		1.0
5		2.0	
	5 (oldest)		0.6

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The small amounts of incorporation in the older leaves could represent either a low rate of synthesis (possibly turnover) of all cutin components or merely the esterification of unhydroxylated palmitic acid into the free hydroxyl groups of the polymer. In order to distinguish between these two possibilities, the hydrogenolyzed products were analyzed by thin layer chromatography, and autoradiograms were prepared from these plates (Fig. 1). The slowest moving component containing the highest amount of ^{14}C is hexadecan-1,7,16-triol derived from 10,16-dihydroxy palmitic acid of the cutin (7). The next higher component has been tentatively identified as hexadecan-1,16-diol derived from ω-hydroxy palmitic acid. The fastest moving component is hexadecanol derived from palmitic acid itself. Results in Figure 1 show that, as the leaves aged, incorporation of radioactivity into all the three major components of the cutin decreased. However, the dihydroxy acid decreased more rapidly than monohydroxy acid, and the nonhydroxylated acid decreased the least rapidly. Silica gel scraped from 1-cm bands along the chromatogram was assayed for ^{14}C in a liquid scintillation counter, and these data confirmed the results of the autoradiography. In fact, in the oldest leaves the small amount of ^{14}C incorporated was essentially all in palmitic acid itself. Thus, this ^{14}C probably represents esterification of the palmitic acid-1-^{14}C fed to the leaves onto free hydroxyl groups present in the already synthesized cutin. The incorporation of label in the younger leaves involves hydroxylation, which is the key process in the synthesis of monomers used in the biosynthesis of the polymer. These polyhydroxy acids, which form the major structural component of cutin, are undoubtedly on the internal structures of the polymer. Free hydroxyl groups are probably present on the periphery of cutin, and the only significant addition to the polymer in the older leaves is esterification on such hydroxyl groups by fatty acids. The results presented in this communication clearly demonstrate that cutin synthesis is under strict physiological control: either the synthesis of the cutin synthesizing enzymes or their activity must be blocked as the leaf reaches full expansion and has no new area to be protected by the cuticle. Since the hydroxylation reactions appear to be the key to cutin synthesis, the hydroxylating enzymes and possibly the esterifying enzymes may be involved in the control of cutin synthesis. It may be recalled that cuticular wax synthesis was also most rapid at the most rapidly expanding stage of development of *Brassica oleracea* leaves (4). Thus, a whole series of enzymes must be regulated in such a way that the rapid expansion is associated with cuticle synthesis, but it essentially ceases at full expansion. It is common knowledge that younger foliage absorbs chemicals much more rapidly than the older ones. The cuticle, no doubt, is an important factor in controlling absorption, and its development is probably responsible for the above observations. Efforts are underway in this laboratory to elucidate the mechanism of synthesis of cutin with the use of labeled precursors and cell free preparations.

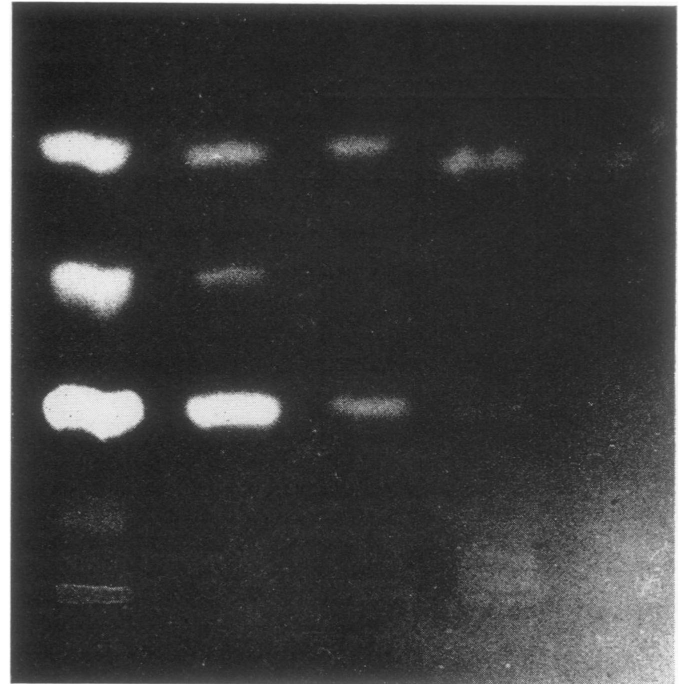

FIG. 1. Autoradiogram prepared from the thin layer chromatogram of the hydrogenolysate of the cutin of *V. faba* leaves of different ages shown in experiment 1 of Table I. Thin layer chromatography on 0.5-mm Silica Gel G with ethyl ether:hexane:methanol (8:2:1, v/v) as the solvent system in a lined tank. X-ray film was exposed for 30 days. Top: Hexadecanol; middle: hexadecan-1,16-diol; bottom: hexadecan-1,7,16-triol; left to right: the youngest to the oldest.

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