GENE MUTATION IN BARLEY INHIBITING THE PRODUCTION AND USE OF C_{26} CHAINS IN EPICUTICULAR WAX FORMATION

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1. Introduction

The various lipid classes present in plant epicuticular waxes are most likely synthesized via an elongation–decarboxylation mechanism [1]. In barley the deposition of these long chain lipid molecules on the cuticle surfaces is controlled by at least 59 genes [2]. These *eceriferum (cer)* genes should be useful tools in elucidating details of the wax synthesizing mechanism. Therefore, waxes from the wild type Bonus and selected *cer* mutants have been chemically analysed. One of the more interesting mutants studied thus far is *cer-j*^{59}. The wax on the leaves of this mutant is not in the form of lobed plates as on Bonus but in the form of thin plates appressed to the cuticle and a few large irregularly shaped bodies [3]. This structural change is accompanied by a 34% decrease in total wax per cm^2 surface, by a small increase in the amount of the esters and a large decrease in the primary alcohols [4]. The latter is the predominant wax class in Bonus leaf was [3,4].

The present paper presents the chain length compositions of the wax classes in the leaf waxes of Bonus and *cer-j*^{59}. The results suggest that enzymes involved in epicuticular wax formation require substrates of specific chain lengths. The hypothesis is presented that more than one enzyme or set of enzymes is responsible for the formation of the individual chain lengths within a wax class. These enzymes may differ only in their chain length specificities and/or belong to different pathways.

2. Materials and methods

Seeds of barley (*Hordeum vulgare* L.) cv. Bonus and the mutant *cer-j*^{59} induced in this line were planted and grown for eight weeks, that is, to heading, in the phytotron at the Royal College of Forestry, Stockholm. The methods used for plant culture, isolation of the leaf wax, separation of the wax into the various classes by preparative thin layer chromatography, preparation of derivatives and determination of the chain length compositions of the wax classes by gas–liquid chromatography have been previously published [4] except for the following.

The free fatty acids were separated from the rest of the wax before thin layer chromatography by passing the wax containing extract through a column having an inner diameter of 2.4 cm packed to a height of 5 cm with 10% NaOH/Gas Chrome Q, 100/120 mesh. Subsequent to elution of the other wax classes with 750 ml petroleum ether (b.pt. 60–80°C), the bound fatty acids were recovered by suspending the column packing in water, acidifying with 10 N H_2SO_4 and extracting with three volumes diethyl ether. After washing the diethyl ether extract with water and drying over sodium sulfate, methyl esters of the fatty acids were prepared using the procedure of Appelqvist [5]. Analysis of the chain length composition of these methyl esters as well as of the aldehydes was accomplished using a Varian Aerograph Model 1700 Gas Chromatograph with flame ionization detectors coupled with an Infotronics Model CRS-100 digital

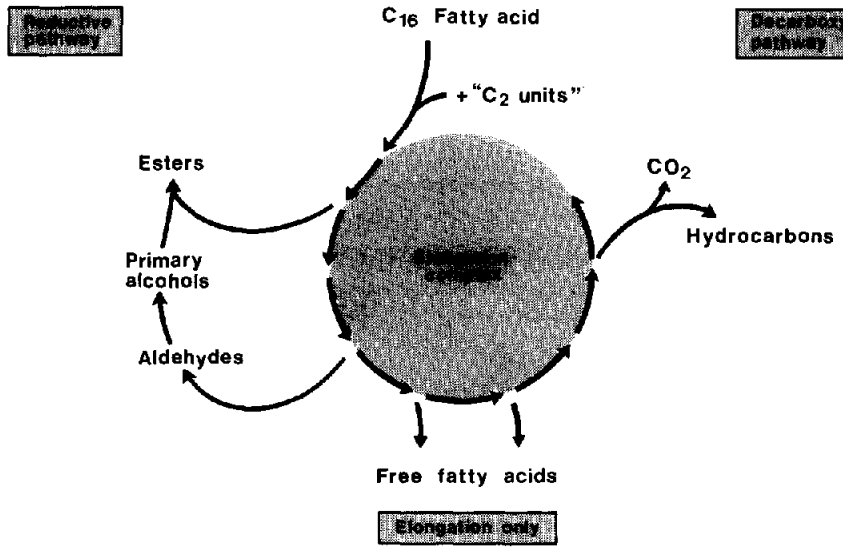

Fig. 1. Diagrammatic presentation of the biosynthesis of the various epicuticular wax classes found on barley leaves. The elongation complex successively adds C_2 units to palmitic acid to form the elongated fatty precursors. The latter may enter the reductive pathway, the decarboxylative pathway or be dissociated from the complex.

electronic computer. Two 152.4 X 0.22 cm stainless steel columns containing 7% SE-30, GC grade on Chromosorb W (DMCS, mesh 60/80) were used. Isothermal and programmed chromatograms were run using column temperatures from 140 to 290°C and nitrogen, helium and air streams adjusted to yield optimum separations. The chain length of the aldehydes was established using internal standards isolated from sugar cane wax [6].

3. Results and discussion

In fig. 1 the general biosynthetic relationships of the five major wax classes composing the epicuticular wax on barley leaves [4,7] are shown. The diagram is based on the elongation–decarboxylation scheme proposed by Kolattukudy (see ref. [8]). The wax classes are divided into three groups according to the reactions occurring after the C_{16} or C_{18} fatty acid precursors have been elongated to specific lengths: 1) primary alcohols, esters and aldehydes formed via the reductive pathway; 2) hydrocarbons formed via the decarboxylative pathway, and 3) free fatty acids formed via dissociation from the elongation complex. Subsequent to synthesis the wax components must be transported to the cell wall, if not synthesized there, and passed through it onto the outer surface of the cuticle. The movement most likely involves additional factors which are equally important in determining the final composition of the epicuticular wax.

The chain length compositions of the six wax fractions isolated from leaves of heading plants of the wild type Bonus and its mutant *cer-j*^{59} are presented in table 1. The major effect of the mutation *cer-j*^{59} is seen in the free and ester primary alcohols, aldehydes and free fatty acids. Compared to Bonus wax these fractions from *cer-j*^{59} have little of the C_{26} chain length. Instead, the wax on *cer-j*^{59} is characterized by dominating amounts of C_{22} and C_{24} in the free fatty acids and the two alcohol fractions and of C_{22}, C_{24} and C_{28} in the aldehydes. The *cer-j*^{59} mutation also modifies the chain length composition of the other two wax fractions – ester fatty acids and hydrocarbons. The ester acid moieties in Bonus consist of C_{16}, C_{18}, C_{20} and C_{22} chain lengths, while in *cer-j*^{59} they are dominated by the C_{22} chain length. This preferential esterification of the C_{22} acid moiety with the C_{22} and C_{24} alcohol moieties dominating in *cer-j*^{59} may be a consequence of the absence of major amounts of the C_{26} alcohol

Table 1
Per cent composition of the wax fractions isolated from leaves on heading plants of Bonus barley and its mutant *cer-j*^{59}

Chain length	Aldehydes		Free primary alcohols		Ester primary alcohols		Free fatty acids		Ester fatty acids		Hydrocarbons	
	Bonus	*cer-j*^{59}	Bonus	*cer-j*^{59}	Bonus	*cer-j*^{59}	Bonus	*cer-j*^{59}	Bonus	*cer-j*^{59}	Bonus	*cer-j*^{59}
14									1.1	0.9		
16									12.8	9.7		
17									1.0			
18						0.3	0.7		13.6	7.9		
19												
20		0.7	0.5	5.6	2.0	3.0	1.6		27.4	17.9		
21	0.1	0.1		0.2	1.3	3.0						4.5
22	0.5	6.9	5.3	50.0	10.3	50.9	14.4	33.1	29.7	57.1		
23	0.8	0.4		0.2			2.5				4.1	9.7
24	1.5	16.1	5.2	27.6	5.3	25.7	12.5	33.3	3.6	2.4	1.2	0.3
25	0.4	0.6									15.9	22.7
26	49.0	24.8	87.0	14.9	80.2	16.9	17.3	2.7	6.7		0.2	
27	0.8	·0.7									2.4	16.1
28	36.2	45.0	2.0	0.8	0.4	0.1	16.0	13.1			0.1	0.1
29	0.4	0.3									4.0	20.8
30	5.4	3.2		0.5			16.6	5.2				
31	0.7	0.1									5.0	8.5
32	1.8	1.1					12.1	4.6				
33	1.0										66.9	17.4
34	1.3						6.2	2.6				
35												
36							0.1	0.8				
x								4.6	4.2	4.1		

· = ⩽ 0.1%
x = Unkowns.

moiety in the latter. It is possible that in Bonus the C_{26} alcohol moiety is preferentially esterified with the shorter fatty acid moieties and the C_{22} alcohol moiety with the longer chain fatty acid moieties. This will be resolved by determining the composition of each individual ester with regard to the different acid and alcohol moieties composing it [9]. The hydrocarbons are a minor component of barley leaf wax [4]. The C_{33} chain length is present in the largest amount in wax isolated from Bonus whereas the shorter C_{25}, C_{27} and C_{29} hydrocarbons dominate in *cer-j*^{59} wax.

In fig. 2 the data are summarized schematically and the probable sites of the major and minor blocks occurring in epicuticular wax formation indicated. The primary block produces an inhibition of the production of the C_{26} chain length and its use in the synthesis of the free fatty acids, aldehydes and free and ester primary alcohols. Since some C_{26} is found in these wax fractions, *cer-j*^{59} is classified as a leaky mutation. Hexacosanoic acid does not accumulate. The relatively large amounts of C_{22} and C_{24} free fatty acids in *cer-j*^{59} suggest that a block occurs in the elongation of C_{24} to C_{26}. However, the presence of chain lengths greater than C_{24}, especially of C_{28} free fatty acids and aldehydes, may be interpreted in two ways. First, they may arise because the mutant is leaky and the block in elongation is not complete. Second, the presence of these chain lengths intimates that the block may not be in the elongation mechanism itself. The latter is supported by the effect of the accompanying minor block. That is, a decrease in the amount of C_{33} hydrocarbon synthesized from a C_{34} fatty precursor via the decarboxylative pathway. Together these results suggest that a common gene

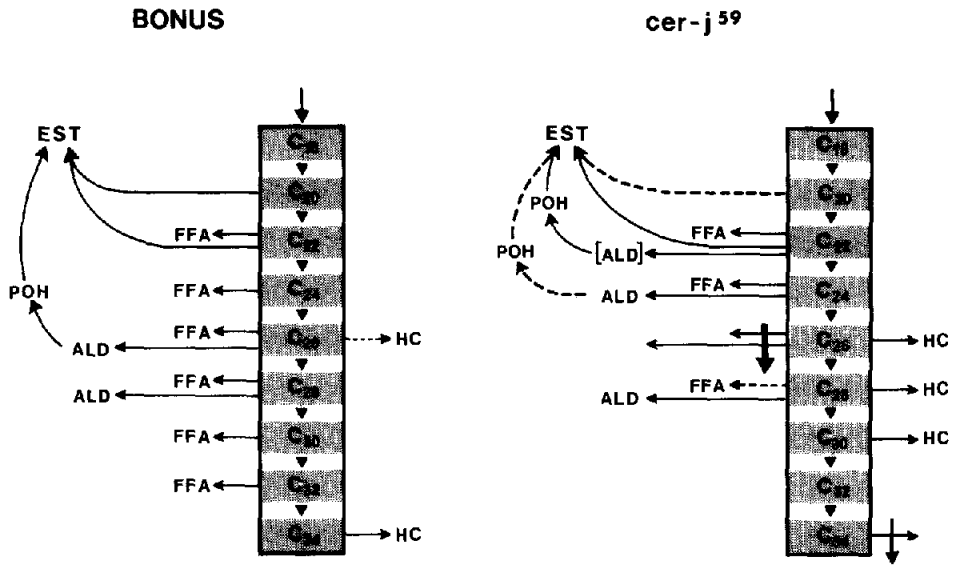

Fig. 2. The major chain lengths found in leaf waxes on heading plants of Bonus and mutant *cer-j*^{59} barley are presented schematically in relationship to their proposed synthesis from the elongated fatty precursors (see fig. 1). Symbols: solid line, major chain lengths; dashed line, important secondary chain lengths; brackets, non-accumulating intermediates; heavy arrow, major block in *cer-j*^{59}; light arrow, minor block in *cer-j*^{59}; HC, hydrocarbons; FFA, free fatty acids; ALD, aldehydes; POH, primary alcohols; EST, esters.

product is involved in utilizing the elongated C_{26} and C_{34} fatty precursors in the subsequent synthesis or transport of these chain lenghts during formation of the various epicuticular wax classes in barley. Thus the major and minor blocks in *cer-j*^{59} are marked outside the elongation complex in fig. 2.

The data presented here demonstrate that enzymes involved in epicuticular wax formation apparently require substrates of specific chain lengths. In addition to the examples of the ester fatty acids given above, consider the synthesis of the primary alcohols from their probable aldehyde precursors [1]. The reductive pathway enzyme(s) cannot substitute a C_{28} precursor for a C_{26} one when the availability of the C_{26} precursor is limited as in *cer-j*^{59}. A specificity for chain length of these enzymes is also been in the recent work of Buckner and Kolattukudy [10] who studied wax formation on young pea leaves using thiol inhibitors in conjunction with tracer studies. They observed that dithioerythritol caused a disproportionally large increase in the formation of aldehydes of the C_{32} chain length which accumulated in the wax. The shorter C_{26} and C_{28} fatty precursors, however, continued to yield the corresponding alcohols as in the control experiments. In the present study the most striking chain length specificity is the inhibition of the production and use of the C_{26} chain length in *cer-j*^{59}. Instead, a considerable increase of the C_{22} chain lengths and a secondary increase of the C_{24} ones occurs in the two alcohol fractions. The selective inhibition of C_{26} and the greater increase of C_{22} than C_{24} suggests that more than one enzyme or set of reductive enzymes is responsible for the formation of the various members of the n-alcohol series, for example, present in Bonus barley leaf wax. That is, the mutation in *cer-j*^{59} affects enzymes synthesizing the C_{26} alcohol but not those synthesizing the other alcohols which continue to be formed as in Bonus. Two possibilities may be envisaged: either a common elongation followed by a series of enzymes differing only in their chain length specificities and/or separate pathways including elongation. An example of the latter situation is the recent demonstration that among the spinach chloroplast fatty acids the synthesis of linolenic acid can occur via a different elongation pathway than that yielding oleic and linoleic acids [11–13].

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References

[1] Kolattukudy, P. E. and Walton, T. J. (1972) Progr. Chem. Fats Other Lipids 13, 119–175.
[2] Lundqvist, U. and Von Wettstein, D. (1973) Barley Genetics Newsletter 3, 110–112.
[3] Lundqvist, U., Von Wettstein-Knowles, P. and Von Wettstein, D. (1968) Hereditas 59, 473–504.
[4] Von Wettstein-Knowles, P. (1971) in: Barley Genet. 2, Proc. Int. Symp., 2nd, P. 1969 (Nilan, R. A., ed), pp. 146–193, WSU Press, Pullman, Washington.
[5] Appelqvist, L. A. (1968) Ark. Kemi 28, 551–570.
[6] Kranz, Z. H., Lamberton, J. A., Murray, K. E. and Redcliffe, A. H. (1960) Aust. J. Chem. 13, 498–505.
[7] Von Wettstein-Knowles, P. (1972) Planta 106, 113–130.
[8] Kolattukudy, P. E. (1968) Science 159, 498–505.
[9] Aasen, A. J., Hofstetter, H. H., Iyengar, B. T. R. and Holman, R. T. (1971) Lipids 6, 502–507.
[10] Buckner, J. S. and Kolattukudy, P. E. (1973) Arch. Biochem. Biophys. 156, 34–45.
[11] Jacobson, B. S., Kannangara, C. G. and Stumpf, P. K. (1973) Biochem. Biophys. Res. Commun. 51, 487–493.
[12] Kannangara, C. G., Jacobson, B. S. and Stumpf, P. K. (1973) Biochem. Biophys. Res. Commun. 52, 648–655.
[13] Jacobson, B. S., Kannangara, C. G. and Stumpf, P. K. (1973) Biochem. Biophys. Res. Commun. 52, 1190–1198.