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COMPOSITION AND STRUCTURE OF MAIZE EPICUTICULAR WAX ESTERS

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Key Word Index *Zea mays*; Gramineae; maize; epicuticular wax esters; structural determination.

Abstract—The structural composition is reported of epicuticular wax esters from maize. The waxes from wild type (*Gl*) plants at different stages of growth and those from some *glossy* (*gl*) seedlings and an albino strain of maize have been analysed for their content of esters. Influence of age and mutations on the epicuticular wax ester composition is discussed.

INTRODUCTION

The epicuticular waxes of wild type seedlings of *Zea mays* L. (*Gl*) and *glossy* (*gl*) mutants are mixtures of long chain aliphatic compounds comprising *n*-alkanes, alcohols, aldehydes, free acids and esters.

The availability of the non allelic gene mutants, simple recessive *gl*1, *gl*2, *gl*3, *gl*4, *gl*5, *gl*7, *gl*8, *gl*15, *gl*16, *gl*18, of an albino strain and of some double mutants provided an opportunity to examine in detail the epicuticular wax biosynthesis in maize [1–3].

The mutants have been shown to control the basic elongation pathway to the fatty acyl chains normally in the range C_{18}–C_{32} arising from the C_2 or C_{16} primers and the following reactions on the activated acyl substrates: (i) release as free acids, (ii) reduction to aldehydes, (iii) reduction to alcohols, (iv) decarboxylation to alkanes, (v) esterification with alcohols.

As this paper is concerned with wax ester composition, it might be useful to report here briefly on the most widely accepted scheme of biosynthesis of this class of compounds [4–7]. The three following esterification processes have been shown to be operative: (i) esterification of fatty acids with fatty alcohols, (ii) direct transfer of acyl groups from phospholipids to fatty alcohols and (iii) acyl transfer from acyl-CoA to fatty alcohols. Furthermore, the data obtained from several studies [4–6] on the alcohol-acid moieties composition of wax esters, indicated the possibility that fatty acids, more than fatty alcohols, making up the ester molecules could originate in pools distinct from those producing the classes of free fatty acids and alcohols.

Our own previous research work on epicuticular wax biosynthesis in maize, has produced results that we consider of relevance to the understanding of ester production in plants. Thus, in *glossy* seedlings, we found that the extent of wax production varied from 20 to 76% of the wild type. Changes were also observed with respect to wax composition that in wild type seedlings is 63% alcohols, 20% aldehydes, 16% esters, 1% alkanes and traces of free acids. Instead, with the exception of *gl*5 in which the dominant class was aldehydes (83%) [8], all maize mutations studied accumulated larger amounts of esters than the wild type plants, in some instances altering also the ratios and homologue compositions of the other wax components.

Ontogenic variations of wax chemistry were also shown by comparison of waxes from wild type seedlings and adult plants. In particular, wax production in maize appeared to be reduced on aging [9]. To account for these observations it was proposed that ester biosynthesis was unaffected by mutations and separated from the site of production of the other wax classes [10].

These findings were considered pieces of evidence leading to the conclusion that at least two elongation–decarboxylation (ED) enzyme systems, defined ED-I and ED-II are active in maize. ED-I would be operative at the seedling stage governing the production and composition of alkanes, aldehydes, alcohols and acids; ED-II would be active for the whole of plant life determining the synthesis of esters. Because of the availability of improved methods and techniques for the analysis of low volatility compounds, the wax esters can now be analysed directly by capillary column GC/MS [11, 12] without the need for a previous alcoholysis reaction in which structural information on individual ester molecules is lost.

In a previous paper [13] we have shown that GC/MS in a repetitively scanning mode gave reliable qualitative and quantitative results. Further work in our laboratory has confirmed the reliability of this technique [14]. This prompted us to re-examine firstly all the ester fractions of maize wild type and mutant waxes previously studied by GC and, secondly, to obtain by means of GC/MS a complete picture of the acid and alcohol components present in each GC peak of the long chain esters whose carbon number ranged from 38 to to 60 and possibly over.

This paper extends the data previously reported on the nature of esters in maize waxes [1, 15]. The new and complete set of data were also examined with the aim to explore whether ester compositions could be used for a better understanding of the activity of the ED-II enzyme

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Table 1. Ester composition (%) of wildtype maize plant at the three different stages of growth of seedlings, young GC and TIC)

	Seedlings		Young plants		Mature plants		*gl1*		*gl2*		*gl3*	
C	GC	TIC*	GC	TIC*	GC	TIC*	GC	TIC	GC	TIC	GC	TIC
36							tr	tr				1
37												
38			2	3			2	3	2	3	1	2
39							tr	tr				
40			8	8	tr		12	15	7	11	9	11
41			tr		tr		tr	1	tr	1	tr	
42	tr	1	11	10	7	9	18	19	9	14	15	16
43	tr		tr		1		1	1	tr		1	
44	tr	1	14	11	30	31	25	23	13	16	22	23
45	tr		tr		2		1	1	tr	tr	1	
46	1	2	10	9	23	24	18	17	11	14	21	19
47	tr		tr		1		1	tr	tr	1	tr	
48	3	5	14	15	11	19	8	7	7	8	13	12
49			tr		1		tr	tr	tr	tr	tr	
50	2	4	4	6	6	10	3	3	5	5	8	7
51			tr		tr				tr	tr	tr	
52	6	10	4	7	4	7	3	3	5	4	6	5
53			tr		1				tr			
54	32	30	15	14	4†		3	3	6	5	2	3
55			tr		3†				tr			
56	39	31	15	12	2†		3	3	12	7	1	1
57			tr		2†				1			
58	12	14	3	4	1†		2		19	9	tr	tr
59			tr		1†				tr			
60	5	1	tr	1					3	1		
62		tr										
64		1										

*Odd ester peaks in TIC too low to be evaluated.
†Overestimated because of the presence of triterpenol esters.

system in maize and, possibly, to disclose specific effects of mutations on it.

RESULTS

The GC and total ion chromatogram (TIC) data of wild type plants (stage of growth: seedlings, 4–5 leaves; young plants, 8–9 leaves; mature plants 12–14 leaves) and those of several *glossy* mutants at the seedling stage of growth are shown in Table 1. Comparison of GC data with the TIC data shows a very good agreement, this reinforcing the validity of the present method for analysing long chain wax esters.

We will consider first wild type plant esters (Table 1). It is apparent that seedlings show the highest specificity for the synthesis of long chain length components, C_{54}, C_{56} and C_{58} representing 83% of the total ester fraction. In contrast, the adult plants produce preferentially shorter chain esters: the two even chains C_{44} and C_{46} make up 53% of the total. A loose chain length specificity is indicated by a relevant 18% of chains in the range C_{52}–C_{60}. Appreciable amounts of odd chain length esters are also present at this stage of growth. As might be expected, the compositional pattern of esters of young plants lies between those of the two extreme stages of growth considered above. As shown in Table 1, the chain length data clearly evidence a pattern with two peaks, the first in the range C_{42}–C_{48} (49%), the second in the range C_{54}–C_{58} (33%) that correspond nicely to the chain length profiles defined by the esters of adult plants and seedlings, respectively.

The percentage distribution of the esters of mutants can be grouped according to three well defined patterns showing a strict similarity with that found for the wild type ester fractions (Table 1). Thus, albino esters match perfectly those of wild type seedlings, mutants *gl2*, *gl4*, *gl8* and *gl16* present ester chain patterns characterized by two peaks (C_{44}; C_{54}–C_{56}) as found for the young plants, and finally *gl1* and *gl3* ester compositional curves are practically superimposable with that of the adult plants.

Quantitative analysis of different weight mixtures of standard ester isomers based on the formula of Aasen *et al.* [16] and on the integration of all the scans due only to the most abundant $[RCO_2H_2]^+$ ion for each peak gave very comparable results [14].

Data on maize wax ester composition, evaluated by the application of the last method of calculation, are given in Tables 2 and 3. Furthermore, GC/MS data are compared with the isomeric compositions calculated from the results of the GC analyses of the methanolysis products of esters assuming a random combination of the acids and alcohols. The methanolysis reaction of ester samples was

and mature plants, and of some *glossy* mutants (*gl*) and an albino strain (analyses by

gl4		*gl5*		*gl8*		*gl16*		Albino	
GC	TIC	GC	TIC	GC	TIC*	GC	TIC*	GC	TIC*
		1	1	1	1	tr	1		
1	1	8	9	6	11	5	8	1	1
tr		1	1			tr			
4	4	18	15	12	18	8	12	2	2
tr		1	2	tr		tr			
8	7	21	18	19	21	11	15	3	5
tr		1	2	tr		tr			
9	11	14	14	15	13	12	16	4	6
tr		1	2	tr		tr			
6	8	9	10	12	10	6	6	3	5
tr		tr	1	tr		tr			
7	7	5	5	6	5	5	4	1	1
tr		tr	1	tr		tr			
12	12	3	4	8	5	9	6	3	4
tr		tr	tr	tr		tr		tr	
17	15	5	5	11	7	13	9	24	22
		tr	tr	tr		tr		tr	
19	17	9	7	8	7	15	12	35	30
		tr		tr		tr		tr	
13	13	3	2	2	2	13	9	19	19
tr									
4	4	tr	1		tr	3	2	4	4
	1							1	1

carried out in this work according to a previously reported method [17].

The majority of ester chains of seedlings are generally made up of one or two isomeric esters and in the range C_{48}–C_{60} the dominant alcohol component is C_{32}. Also data related to esters of young plants are reminiscent of those of seedlings. In fact, in chains C_{48} through C_{60} the principal ester isomer is again that with the alcohol moiety C_{32} (Table 2).

The young plant ester chains, except the longest C_{54}–C_{60}, show a general loose isomeric specificity, having an average of four to five isomers in each homologous ester chain. Furthermore, in the range C_{38}–C_{46} there are at least three dominant isomers in each chain.

The adult plant esters, similar to the young plant esters, show a wide number of isomeric esters within each chain. However, in contrast to both seedlings and young plants, these isomers show no pattern related to any particular alcohol chain: instead, in few instances, it can be noticed how the partial dominance of certain ester isomers is brought about by the acid component such as acids C_{20} and C_{22} in the ester chains C_{46}, C_{48}, C_{50} and C_{52}.

Discrete amounts of triterpenol esters, which co-elute with linear esters, have been found in waxes from mature plants (Tables 1 and 2). Analysis with GC/MS gave structural indications for the presence of amyrin series components. A very intense ion at *m/z* 218, typical of a retro-Diels–Alder fragmentation of such compounds [18], is in fact evident. Due to the reciprocal contamination of the two types of esters their quantitative evaluation has not been performed.

Data related to mutants are given in Table 3. The acid-alcohol compositional picture of ester chains C_{38}–C_{46} shows no particular trends differentiating among the mutants. It appears that the major components of these ester chains are those comprising acid and alcohol homologues in the middle of the two ranges C_{14}–C_{28} and C_{18}–C_{32}, respectively.

Interestingly, the acid-alcohol composition of esters C_{48}–C_{60} evidences two well differentiated groups of mutants. In fact, mutants *gl5*, *gl8* and albino have ester chains in which the dominant isomer comprise the alcohol moiety C_{32}; mutants *gl2*, *gl4* and *gl16* are, on the other hand, characterized by the shorter homologous alcohol triacontanol (C_{30}). Finally, while *gl1* resembles more the first group of mutants, *gl3* can hardly be associated with either of the two defined groups.

Homologue esters with odd carbon numbers are either absent or present in traces up to 2% (Table 1). Preliminary analytical data available from this study (GC/MS), indicated that odd esters were made up by both combinations of even and odd fatty acids with odd and even

Table 2. Isomer composition (%) of the esters of maize normal plants at the three stages of growth of seedlings, young and mature plants (analyses by MS† and GC‡)

Ester and acid-alcohol components (carbon no.)		Seedlings		Young plants		Mature plants		Ester and acid-alcohol components (carbon no.)		Seedlings		Young plants		Mature plants	
		MS	GC	MS	GC	MS	GC			MS	GC	MS	GC	MS	GC
38	16–22			21				**50**	16–34			12		6	
	18–20			49					18–32	66	82	93	93	8	
	20–18			30					20–30	34	10	23	5	26	
									22–28		8	4	2	32	15
40	16–24		25	30	35	60			24–26		*		30	17	85
	18–22	45	15	13	9	20			26–24			3	39	4	14
	20–20	55	37	49	46	20	100		28–22				1		2
	22–18		23	8	10				30–20				5		1
42	16–26		9	14	3	22		**52**	26–32	89	94	86	72	83	23
	18–24	35	11	31	14	33			22–30	11	5	10	18	08	
	20–22	36	22	28	24	42	93		24–28		1	2	1	28	13
	22–20	29	25	25	57	3	7		26–26			2	3	11	40
	24–18		33	2	2				28–24				4		20
									30–22				1		3
44	16–28		5	4	1	4			32–20						1
	18–26		8	12	2	8									
	20–24	68	35	62	51	82	89	**54**	22–32	100	97	98	99	*	30
	22–22	32	28	18	33	6	10		24–30		3	2	1		
	24–20		24	4	13		1		26–28				2		53
									28–26				1		18
46	16–30		18	14	2	6			30–24				1		36
	18–28		3	4	1	4			32–22						3
	20–26	45	21	36	7	59	39								
	22–24	55	37	41	82	29	56	**56**	24–32	100	100	100	98	*	26
	24–22		21	4	8	2	4		26–30				1		
							1		28–28				1		16
48	16–32	76	83	84	37	12			30–26				1		33
	18–30	5	3	3	1	5			32–24						33
	20–28	5	2	3	1	20	11								
	22–26	8	5	6	26	50	43	**58**	26–32	100	100	100	98	*	39
	24–24	6	7	4	30	13	41		28–30				1		
	26–22				4		4		30–28				1		16
	28–20				1		1		32–26						45
								60	28–32	100	100	100	98	*	55
									30–30				1		
									32–28						45
								62	30–32	100	100				100

*Not computerized because contaminated by large amount of triterpenol esters.

†Based on the intensity of the $[RCO_2H_2]^+$ ion.

‡Isomeric compositions expected assuming that the acids and alcohols were esterified randomly (see Text).

alcohols, respectively. Odd esters comprising even acids esterified with odd alcohols were the most prevalent.

DISCUSSION

In maize the wax esters are the only class of compounds whose production is not negatively affected by mutations or by plant age. In fact, while they amount to 16% of the total wax from wild type plants [10], they become even more abundant in the surface lipids from *glossy* mutants, reaching up to 71% in *gl1* [3]. It has been proposed earlier that they are synthesized by an enzymatic system (ED-II) whose activity is independent from that of (ED-I) governing production of all the other wax classes.

The present study reports on compositional changes adopted by wild type plants and mutants to ensure production of the seemingly most necessary long chain aliphatic esters during ontogeny and also in response to mutations. Thus, it is evident that the plant ester composition changes dramatically with aging even in the relatively short period of time between the seedling and the young plant stage. In this respect a relevant point is that in wild type seedlings the alcohol most utilized in ester synthesis is the same C_{22} compound we find is dominant

in wax free alcohols. However, this is not surprising because there is no reason why an organism should diversify an enzyme system preposed to the synthesis of a certain compound, e.g. alcohol C_{32}, though utilized in two distinct metabolic compartments. The data shown in Table 3 seem to confirm this thesis. Mutants *gl*2, *gl*4 and *gl*16 whose major effect on ED-I classes of compounds was a chain length shortening of a C_2 unit (from C_{32} to C_{30}) [1, 8] are in fact, characterized in esters C_{46} to C_{60} by the alcohol C_{30} that in combination with the related acids make up the dominant isomers. The data of the albino mutant reported in Table 3 complete the picture: the composition of the ester chains common to both albino and wild type seedlings are practically phenotypic copies as were the homologues found in the other wax classes [17].

The mass spectral compositional data presented in Tables 2 and 3 show two further features: (i) the range of esterified acids is different from that of the alcohols that determine the most abundant isomers; (ii) in many homologous chains, several possible combinations to produce expected esters are missing. The latter finding is in most

Table 3. Isomer composition (%) of esters of glossy mutants and an albino strain of maize (analyses by MS* and GC*)

Ester and acid-alcohol components (carbon no.)		*gl*1		*gl*2		*gl*3		*gl*4		*gl*5		*gl*8		*gl*16		*Albino*	
		MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC
32	16–16										100		100				
34	16–18		100				100		100		69		70				100
	18–16										31		30				
36	16–20		77		100		82		80		67		65				45
	18–18		23				18		20		11		14				43
	20–16										28		21				12
38	14–24	2				4								10			
	16–22	20	20	20	27	28	40		39	31	24	33	40	26			45
	18–20	50	42	47	73	47	26		37	55	34	45	28	40			43
	20–18	28	38	33		21	34		24	14	26	22	23	24			12
	22–16										16		9				
40	14–26					1											
	16–24	34	13	24	28	39	49	29	33	33	23	36	34	36		45	52
	18–22	11	9	20	11	10	8	14	15	11	8	12	15	11		19	21
	20–20	46	58	44	61	44	33	49	42	50	54	45	42	43	100	36	18
	22–18	9	20	12		6	10	8	10	6	12	7	8	10			8
	24–16										3		1				
42	16–26	16	11	15	8	32	19	14	8	21	8	13	7	15		31	25
	18–24	32	10	29	26	24	27	31	26	26	15	33	24	28		21	41
	20–22	24	20	26	21	23	27	26	31	21	25	27	40	26	33	23	14
	22–20	26	50	27	45	19	23	25	31	32	46	25	27	31	67	25	18
	24–18	2	9	3		2	4	4	4		5	2	2				2
	26–16										1						
44	14–30							3						3			
	16–28	3	2	15	9	16	4	8	13	5	3	3	2	12		52	47
	18–26	11	11	15	7	11	8	12	5	15	5	15	55	9		14	15
	20–24	60	32	47	45	55	65	53	46	49	49	59	60	49	63	25	22
	22–22	19	23	18	15	15	14	19	20	23	23	22	25	21	28	9	11
	24–20	6	32	4	24	3	7	5	13	8	19	5	8	6	9		4
	26–18	1		1			2		3		1						1
46	14–32	1										4					
	16–30	2		25	22	28	5	37	17	6		7	2	43		18	18
	18–28	2	2	11	7	5	2	4	12	4	3	3	2	4		59	39
	20–26	33	40	27	12	31	29	23	12	35	21	31	20	16	13	9	12
	22–24	53	41	28	31	31	52	29	37	49	56	49	62	32	72	14	25
	24–22	5	17	4	8	3	7	3	11	6	12	6	12	4	6		4
	26–20	3		3	20	2	4	4	9		8		2	1	9		2
	28–18	1		2			1		2								

*See footnotes † and ‡ in Table 2.

Table 3.(*Continued*)

Ester and acid-alcohol components (carbon no.)		*gl1*		*gl2*		*gl3*		*gl4*		*gl5*		*gl8*		*gl16*		*Albino*	
		MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC	MS	GC
48	14–34	1															
	16–32	30	4	8		38	5	22	4	50	4	60	13	28		70	24
	18–30	2		23	18	9	5	37	17	4		8	2	22		16	14
	20–28	6	9	25	12	14	13	11	28	5	14	6	12	13	16	7	29
	22–26	34	55	22	8	27	33	13	11	23	33	17	28	27	35	7	13
	24–24	21	31	9	16	12	36	10	22	18	40	9	39	10	31		8
	26–22	2		2	6		5	7	8		7		4		12		1
	28–20	4		11	40		3		10		2		2		6		
50	16–34	19								9							
	18–32	23	8	10		14	5	16	4	45	7	55	23	20		61	36
	20–30	4	3	45	43	35	26	63	39	5		20	16	56	62	16	13
	22–28	14	21	24	12	15	15	11	25	8	30	10	19	24	12	23	41
	24–26	24	68	10	6	26	23	10	6	33	32	15	21		4		5
	26–24	11		6	18	10	26		16		30		17		19		4
	28–22	3		5	18		4		9		1		4		3		1
	30–20	2			3		1		1								
52	18–34	16															
	20–32	49	60		1	29	24	20	11	95	44	80	69	23	9	90	44
	22–30	13	7	58	37	28	28	71	42	5		20	12	66	78	10	25
	24–28	7	33	18	8	15	9	9	18		27		7	11	2		23
	26–26	15		10	6	20	15		6		22		4		4		4
	28–24			14	47	8	22		22		7		8		7		4
	30–22				1		2		1								
54	20–34	12															
	22–32	81	94	15	1	65	37	30	17	94	78	100	88	31	34	97	72
	24–30	5	6	45	47	35	26	62	44	6			7	58	53	3	12
	26–28	2		23	15		9	8	23		17		2	11	8		13
	28–26			17	30		19		11		5		3		5		3
	30–24				7		9		5								
56	22–34	28															
	24–32	72	100		1		43	39	14	100	94	100	93	31	11	100	65
	26–30			54	53		32	54	47				4	69	84		13
	28–28			43	44		14	7	36		6		3		5		22
	30–26			3	2		11		3								
58	26–32				1		48	35	16	100	100	100	90	36	28	100	75
	28–30			97	97		45	65	77				10	64	72	25	25
	30–28			3	2		7		7								
60	28–32				18		75	56	63	100	100		100	100	100	100	100
	30–30			100	82		25	44	37								
62	30–32						100		100								

*See footnotes † and ‡ in Table 2.

cases straightforwardly revealed by the poor agreement between the chain-couple distributions defined by the mass spectral analysis and the combinations expected from a random esterification of the acids and alcohols obtained by methanolysis of esters (GC data). This scarce qualitative fit, affecting consequently also the quantitative data, may only in part be due to losses of volatile components during their preparation for GC analysis and, anyhow, should not arise from the mass spectrometry analytical procedure, considering the very close GC and TIC data of Table 1.

In our opinion, the data of the present study indicate (i) that the ED-II system governs possibly esterification both through the chain specificity of the ED-I reductive system giving rise to certain alcohols and (ii) by selecting giving isomers from a wider number of possible ester molecules.

In conclusion, our results support the hypothesis [4–6] that in esterification there is a certain degree of chain length specificity.

EXPERIMENTAL

Wax esters. Ester fractions were from our stock of previously reported experimental work (see ref. [1] for a review).

Analysis. GC and GC/MS analyses were carried out as detailed in ref. [13, 14].

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