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THE *cer-cqu* REGION IN BARLEY: GENE CLUSTER OR MULTIFUNCTIONAL GENE

by

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Among the *eceriferum* loci *cer-c*, *-q* and *-u* control three different steps in the pathway leading to β-diketones, hydroxy-β-diketones and esterified alkan-2-ols. Chemical analyses of the wax on spikes of wild type and representative single and apparent multiple mutants demonstrated that such mutants do not affect the composition of the other wax lipids and confirmed the earlier deduced sites of action of *cer-c*, *-q* and *-u*. Each pair of the three markers *cer-c*^{3}, *-q*^{35} and *-u*^{58} was found by test cross analysis to be closer together than 0.025 map units. This deduction was derived from the failure of the markers to recombine in the 26,933 gametes tested. The obtained results are expected 95% of the time if the actual distance between any pair is approximately 0.0012 map units. The linear sequence of the three markers could not be determined. The combined results lead to the suggestion that the *cer-cqu* region is more likely to represent a multifunctional gene than a gene cluster.

1. INTRODUCTION

The biosynthesis and deposition of waxes on the cuticular surfaces of barley plants are controlled by the *eceriferum (cer)* genes. Figure 1 summarizes the biosynthetic relationships among the β-diketones, hydroxy-β-diketones and alkan-2-ol containing esters which together account for more than half of the wax on wild type Bonus spikes (35). The sites of action of three *cer* genes are indicated in Figure 1. The elongation system(s) leading to these lipid classes differs from the one(s) giving rise to the other

Abbreviations: DEPH = di-2-ethylhexylphthalate, GLC = gas liquid chromatography, TLC = thin layer chromatography.

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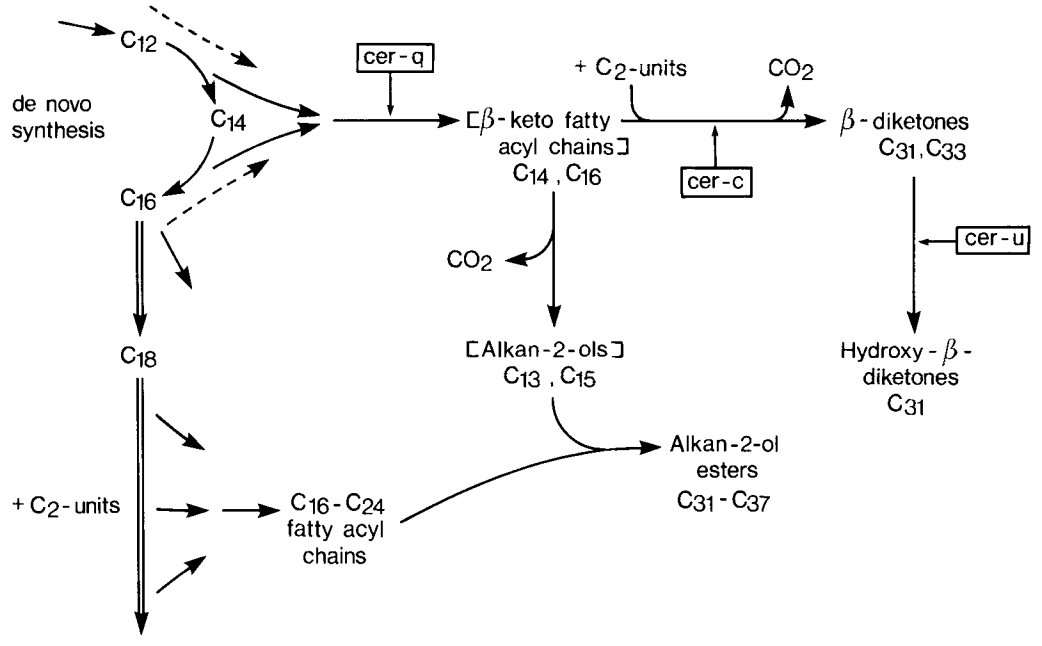

Figure 1. Biosynthetic relationships among the β-diketones, hydroxy-β-diketones and alkan-2-ol containing esters present in barley epicuticular waxes.

The sites of action of three *cer* genes are shown. The elongation system(s) leading to the other wax classes is (are) indicated by the double arrows extending downward from the C_{16} acid.

important barley wax lipids which are believed to arise as follows (see 36): C_{16} fatty acyl chains are elongated by C_2-units from malonyl-CoA. Before arriving on the cuticle surface, the resulting chains may serve as substrates for the different wax lipids. They can enter (i) the decarboxylation pathway which gives rise to the alkanes and when present the secondary alcohols or (ii) the reductive pathway which yields the aldehydes, primary alcohols and alkan-1-ol containing esters. Others of the fatty acyl chains are released from the elongation system(s) as free fatty acids.

During the isolation and allele testing of 1252 *cer* mutants, only 13 appeared to be mutated simultaneously at two or more of the *cer* loci (17, 18, 19, 20, 21, 22). Interestingly, only *cer-c, -q* and *-u* were involved. All possible double as well as the triple mutant combination have been identified. Trisomic analyses revealed that *c, q* and *u* were located on the same end of chromosome 4 (29). None of them were linked with the translocations involving chromosome 4 used for analysis indicating that they must be located toward the end of the chromosome. Combining these observations with the knowledge of their roles in wax biosynthesis (Figure 1) suggested that *cer-c, -q* and *-u* might represent part or all of a gene cluster concerned with wax biosynthesis. This paper presents results of genetic tests aimed at mapping *cer-c, -q* and *-u*. In addition, the chemical composition is reported for the lipid classes mentioned above from spikes of representative single, triple as well as the three types of double mutants. Based on these results the possibility that *cer-c, -q* and *-u* form a cluster comprised of three individual genes determining three individual polypeptides (gene cluster) versus a single gene determining a polypeptide with several functions (multifunctional gene) is discussed.

2. MATERIALS AND METHODS

2.1. Chemical studies

Seeds of barley (Hordeum vulgare L.) cv. Bonus as well as of the *eceriferum* mutants *cer-c^{36}, -q^{42}, -cu^{108}, -cqu^{124}, -qu^{510}, -cq^{645}* and

-qu^{813} (16, 17, 18, 19, 22) were planted and grown to heading (approximately 60 days later) under optimum conditions for vegetative growth (3, 4) in the Phytotron at the Royal College of Forestry, Stockholm (32). The three to four heading spikes were then harvested and wax collected from whole spikes or spikes minus awns as described earlier (33, 37). Leaving the plants under the optimum conditions for vegetative growth resulted in the heading of the next set of spikes eight to nine days later. This process could be repeated an additional two times to give at least 12 to 16 spikes per plant at approximately the same developmental stage from which wax could be collected.

Preparative thin layer chromatography (TLC) on silica gel H (Merck) plates was used to isolate the wax classes as follows: Benzene was employed as the developing solvent to separate the hydrocarbons, esters, aldehydes, primary alcohols and free acids from the wax of the *cer* mutants. When the same system was used with the Bonus spike wax only the hydrocarbons and free fatty acids were recovered. To obtain the other three wax classes, »hybrid« TLC plates impregnated on the lower third with copper acetate (24) were employed. Using benzene as the developing solvent, it was possible to separate the esters, aldehydes and primary alcohols without contamination by the β-diketones and hydroxy-β-diketones which remained at the origin complexed with the copper. All lipid classes were eluted using chloroform with the exception of the free acids which were recovered with acidified chloroform:methanol (23).

To allow an estimation of the relative amounts of the various wax classes to be made, to check the purity of isolated lipid classes as well as to follow the course of and purify the products of chemical reactions, TLC on silic gel H plates was carried out using benzene and/or hexane:diethyl ether:acetic acid (70:30:2, v/v/v) as the developing solvents. The lipids were visualized as described earlier (33).

The isolated lipid classes and/or their derivatives were analyzed via gas liquid chromatography (GLC). A Hewlett-Packard 5840A series gas chromatograph with dual flame ionization detectors coupled to a Hewlett-Packard 5840A GC terminal was used. Three types of stainless steel columns were employed: i) 152 cm × 3.2 mm containing 5% SE30 on 100/120 mesh Anakrom ABS (Analabs, Conn., USA), ii) 152 cm × 3.2 mm containing 3% SP-2100 on 80/100 mesh Supelcoport (Supelco, Penn., USA) and (iii) 91 cm × 3.2 mm containing 1% Dexsil-300 on 100/120 mesh Supelcoport. Temperature programs were run from 140 to 310 °C or from 200 to 340 °C at a rate of 3 °C · min^{-1}. The injector temperature was 260 °C and that of the detectors 350 °C. Nitrogen, helium and air streams were adjusted to yield optimum separations.

Methods used in the preparation and isolation of methyl esters of the free acids and of the acetate derivatives of the free primary alcohols as well as those obtained by reduction of the aldehydes have been given earlier (23, 33). To ensure that contamination did not occur during preparation of the derivatives, aliquots of the primary alcohols and aldehydes were routinely injected into the GLC directly after their initial isolation. Sometimes an additional peak that co-gas chromatographed with a C_{21} alcohol acetate (elution temperature approximately 220 °C) was present after derivatization. This compound was isolated by preparative TLC and analyzed by GLC-mass spectrometry. The base peak of the obtained spectrum was at m/e 149. Strong fragment ions were also observed at m/e 279, 167 and 113. Exact mass measurements of these four ions supported the identification of the compound as a phthalic acid ester. Final identification was made by comparing the obtained mass spectrum with that of authentic di-2-ethylhexylphthalate (DEHP) to which it was identical. The source of the DEHP was found to be the Triflex vinyl gloves (Travenol Laboratories, Norfolk, England). An extract of the gloves yielded sufficient material to demonstrate that it co-chromatographed on the thin layer plates employed with the primary alcohol acetates when benzene was used as the developing solvent. Phthalates have recently been implicated as the interferring substances in GLC analysis of the fatty acid composition of blood plasma drawn through plastic tubing etc. (e.g., 8, 31). When the contaminating phthalate is injected on the SP-2100 column together with standard methyl esters it elutes with methyl docosanate (C_{22}), and when injected with hydrocarbons it elutes with pentacosane (C_{25}).

Transmethylation of the esters (23) was carried out to yield the alcohol moieties and the methyl esters of the fatty acid moieties. These products were recovered by extracting with three small aliquots of hexane after diluting with water and adding NaCl. They were then separated on TLC plates using benzene as the developing solvent. The methyl esters were recovered, the alcohols treated with acetyl chloride (33) and the plate developed a second time with benzene. Thereafter the ester alcohol acetates were recovered.

2.2. Genetical studies

To establish the sequence and distances between *cer-c*[3], *-q*[35] and *-u*[58] (16) the following crossing program was carried out. Pairwise crosses were made among the mutants. This yielded three different F_1 types, each involving two of the three mutants in trans configuration; namely, $c++/+q+$, $c++/++u$ and $+q+/++u$ in which the marker order is arbitrary. Each of these F_1 types has the potential to produce four genotypically different gametes. To illustrate, the F_1 $c++/+q+$ could produce $c++$ and $+q+$ parental type gametes as well as $cq+$ and $+++$ crossing over type gametes. To test how many of each type of gamete is produced, test crosses with a female homozygous for *cer-cqu*[420] (22) were made. All progeny will have a mutant phenotype except those arising from fertilization of the *cqu* egg by the $+++$ crossing over pollen. Thus, the number of wild type progeny is equal to one half of the crossing over gametes. Given that p equals the probability of a cross over, then p/2 is the probability of there being a wild type in the progeny. When the number of progeny equals n, the probability (Pr) of observing x plants with a wild type phenotype follows the binomial distribution which can be written as

$$Pr\{X=x\} = \binom{n}{x}\left(\frac{p}{2}\right)^{x}\left(1-\frac{p}{2}\right)^{n-x} \qquad [1]$$

It is also possible to pool the data from several test crosses and obtain thereby an estimate of the map units between the most distant of the three markers. The following assumptions must be made: a linear, but unknown sequence of the three markers and that the two distances between the three markers are equal. The error introduced by making the latter assumption is insignificant when the distances involved are very small and the number of progeny in each of the three test crosses are of the same magnitude. A wild type gamete has the following probabilities of occurring:

$2\left(\frac{p}{2}\right)$ in the test cross $c++/++u \times cqu/cqu$

$\frac{p}{2}$ in the test cross $+q+/++u \times cqu/cqu$

and

$\frac{p}{2}$ in the test cross $c++/+q+ \times cqu/cqu$

if it is assumed that the marker *cer-q*[35] lies between the two other markers. The joint probability (Pr) of observing x plants with a wild type phenotype among the combined progeny from all three test crosses can be expressed as:

$$Pr\{X=x\} = \sum_{y=0}^{x}\binom{n-n_{cu}}{y}\left(\frac{p}{2}\right)^{y}\left(1-\frac{p}{2}\right)^{n-n_{cu}-y}\binom{n_{cu}}{x-y}(p)^{x-y}(1-p)^{n_{cu}-x+y} \qquad [2]$$

which can be rewritten as:

$$Pr\{X=x\} = \left(\frac{p}{2}\right)^{x}\left(1-\frac{p}{2}\right)^{n-x}\left(1-\frac{\frac{p}{2}}{1-\frac{p}{2}}\right)^{n_{cu}}\sum_{y=0}^{x}\binom{n-n_{cu}}{y}\binom{n_{cu}}{x-y}\left(1+\frac{1}{1-p}\right)^{x-y} \qquad [3]$$

where n_{cu} is the number of progeny in the test cross $c++/++u \times cqu/cqu$.

The above studies were carried out on plants grown in the field and green houses at the Agricultural Research Department, Risø National Laboratory, Denmark, as well as in the Phytotron of the University of Oslo, Norway.

As a basis for evaluation of mutant phenotypes, isolation and determination of the amount of wax on each set of 14 Bonus spikes was carried out as described earlier (34). Immediately after removing the wax from each set of spikes and allowing the chloroform to evaporate, they were weighed. The spikes were obtained from plants 50 to 70 days after sowing in the Stockholm Phytotron. At each harvest time first, second, third and fourth spikes, in order of their emergence, were placed in separate sets of 14.

3. RESULTS

3.1. Lipid class composition of the waxes on spikes of Bonus and seven *cer* mutants

The β-diketones and hydroxy-β-diketones which form 50% of Bonus spike wax (35) were earlier shown to be absent in the spike waxes of *cer-c*^{36}, *-q*^{42}, *-cu*^{108} and *-qu*^{510} (34). In the present study these two lipid classes were also found to be lacking in the spike waxes of *-cqu*^{124}, *-cq*^{645} and *-qu*^{813}. A visual comparison was made of the relative amounts of the other important lipid classes --hydrocarbons, esters, aldehydes, primary alcohols and free fatty acids-- after their separation on thin layer plates in two solvent systems. The relative amounts of these five lipid classes appeared to be similar in all the above mentioned genotypes as well as in *cer-u*^{69} (38) with the possible exception of a slight increase in the amount of the aldehydes in *cer-qu*^{510} and *-qu*^{813}.

Columns were used to separate large amounts of β-diketones and esters from *cer-u*^{69} spike wax in earlier studies (25, 37). Separation and determination of the approximate amounts of the other wax classes were also carried out in one case starting with 142 mg of wax with the following results: β-diketones 50%, hydrocarbons 16%, esters 11%, aldehydes 4%, primary alcohols 3%, free acids 5% and three unknowns 11%. These results show that aldehydes and primary alcohols are minor constituents of *cer-u*^{69} spike wax.

Measurements of the amount of wax per spike for Bonus, *cer-u*^{69} and *-c*^{36} (34) combined with the knowledge of the percentage of β-diketone wax classes in these waxes (34, 35) led to the conclusion that failure to synthesize one or both β-diketone lipid classes in *cer-u*^{69} and *-c*^{36}, respectively, was not accompanied by decreases or increases in the non-β-diketone wax classes (34). Combining the present observations with these earlier results allows one to infer that no substantial alteration in the absolute or relative amounts of the non-β-diketone wax classes per spike is caused by the herein studied *cer* mutations.

3.2. Chain length distributions of the lipid classes on spikes of Bonus and six *cer* mutants

Table I presents the composition of the hydrocarbons present in the wax of seven genotypes. In all cases hentriacontane (C_{31}) predominates, varying from approximately 64 to 74% by weight. Nonacosane (C_{29}) is the second most important homologue and accounts for 15 to 23% of the hydrocarbons. No significant differences can be seen among the hydrocarbon chain length distributions of these seven genotypes which are analogous to that previously reported for spike wax of *cer-u*^{69} (38).

Among the aldehydes (Table II) the C_{32} homologue is present in the largest amount followed by the C_{30}, C_{28} and C_{26} homologues in order of decreasing importance. In Bonus, *cer-q*^{42}, *-qu*^{813} and *-c*^{36} spike waxes, the C_{32} chain length represents 50 to 56% by weight of the aldehydes (analyzed as their alcohol acetate derivatives), whereas in *cer-cqu*^{124} and *-cu*^{108} it accounted for only 39 to 41%. The chain length distributions for the latter two genotypes are similar to that reported for the aldehydes of *cer-u*^{69} spike wax (38); namely C_{32}, 44.2%, C_{30}, 31.0%, C_{28}, 13.8%, and C_{26}, 9.5%. Whether or not these are significant differences is unknown at present.

The C_{26}, C_{28}, C_{30} and C_{32} primary alcohols (analyzed as their acetate derivatives) account for at least 82% by weight of this wax class in all the investigated genotypes (Table III). In contrast to the aldehydes, however, it is the shortest of these homologues, namely C_{26}, that is present in the largest amount, ranging from 37 to 52%. The distribution from the genotype *cer-c*^{36} having the smallest amount of the C_{26} homologue has 17% of the C_{32} homologue which is 5 to 9% more than is present in the distributions of the other six genotypes. The previously studied free primary alcohols from *cer-u*^{69} (38) represent the other extreme in having only 3% of the C_{32} homologue and 57% of the C_{26} homologue. Again as when comparing the aldehyde distributions, it is not possible to say whether the observed variations in the free alcohol distributions are significant. As pointed out in section 3.1, they are both relatively minor constituents of these waxes.

The composition of the primary alcohols on

Table I

Composition of hydrocarbons found on spikes of seven barley genotypes (weight %).

Number of Carbons[a]	Bonus	*cer-* q^{42}	cu^{108}	cq^{645}	qu^{813}	c^{36}	cqu^{124}
21	0.3	0.1	0.2	0.3	0.3	0.3	0.3
22	0.2		tr	0.1	tr	tr	tr
23	1.5	1.5	2.2	1.9	2.1	1.9	1.8
24	0.2	tr	tr	0.1	0.1	tr	0.1
X			0.2	tr	0.1	0.2	0.1
25	2.0	1.4	2.5	3.0	1.9	1.6	1.8
26	0.1	tr	tr	0.1	tr	tr	tr
X	0.1		0.3	tr	0.2	0.4	0.2
27	2.0	1.8	2.3	2.1	2.3	2.1	1.9
28	0.1	tr	0.3	0.3	0.2	0.1	0.2
X	0.1	0.3	0.8		0.7	1.2	0.6
29	14.7	14.9	22.7	17.0[e]	17.1	14.7	15.8
30[b]	1.3	1.4	1.7	1.5	1.7	1.5	1.6
31[c]	72.1	75.1[d]	63.7	69.4	68.6	71.3	71.3
32	1.0		1.2	0.8	1.0	1.1	0.9
33	4.1	3.6	2.1	3.3	3.6	3.6	3.7

a. X represents a member of a second homologous series whose retention times are slightly less than the following normal hydrocarbon.
b. Percent is overestimated as the C_{30} has not been integrated separately from an unknown of approximately the same amount as C_{30}.
c. Includes the longest member of the X series which was too small to be resolved from the C_{31} hydrocarbon under the GLC conditions used.
d. Includes C_{32}.
e. Includes member of X series with slightly shorter retention time.

tr = trace (< 0.1 %)

the spike minus awns was also determined for *cer-c*[36]. The amounts of the five most abundant homologues were as follows: C_{24}, 12.2%; C_{26}, 45.1%; C_{28}, 15.1%; C_{30}, 12.0% and C_{32}, 7.4%. A comparison of this data with that for the whole spikes of *cer-c*[36] (Table III) indicates that the awns make a major contribution to the total amount of the C_{32} alcohol in wax from whole spikes. The decrease in the relative importance of the C_{32} homologue in the wax from the spike minus awns is primarily compensated for by increases in the relative importance of the C_{24} and C_{26} primary alcohols. These conclusions are in agreement with the previous analyses (38) of the wax on the awns, spike minus awns and whole spikes of *cer-u*[69].

In Table IV are presented the chain length distributions of the free fatty acids (analyzed as their methyl ester derivatives) isolated from the spikes of six genotypes. Compared to the wax classes discussed above, each of the observed spectra is characterized by having at least six relatively prominent chain lengths, namely C_{20}, C_{22}, C_{24}, C_{26}, C_{28} and C_{30}. No significant differences are recognizable among these six genotypes or that of the fatty acids from the *cer-u*[69] spike wax analyzed earlier (38).

The separation and quantitation of the esters via the GLC procedures used herein give somewhat different results from the earlier (Table I in 37) analyses in that the proportion of the C_{48} and longer chain lengths is apparently increased. The results presented in Table V are presumed to be the more accurate for the following reasons. Calculation of the expected distribution of *cer-u*[69] ester homologues based

Table II

Composition of aldehydes present in the spike wax of six barley genotypes (weight % as alcohol acetates)[a].

Number of Carbons	Bonus	q^{42}	qu^{813}	*cer-* cqu^{124}	c^{36}	cu^{108}
20	tr	0.2	tr		0.1	
22	0.1	0.3	0.3	tr	0.1	0.5
23						tr
24[b]	0.9	1.1	0.7	3.3	2.6	1.9
25		tr	tr	0.2	0.3	0.3
26[b]	4.1	7.8	7.2	9.9	9.9	7.6
27	0.2	tr	tr	0.5	0.5	0.4
28[b]	8.4	10.6	9.4	13.5	10.1	13.0
29	0.5	0.3	0.5	0.9	0.8	0.5
30[b]	27.0	26.1	22.5	26.4	22.9	35.4
31	1.9	1.1	1.3	1.7	2.1	1.0
32[b]	54.8	51.3	55.8	40.7	49.8	38.8
33	tr	tr	tr	tr	tr	tr
34	2.1	1.1	2.3	2.9	2.9	0.7

a. Datum for *cer-*cq^{645} not available.
b. Includes minor amount of a member of a second homologous series whose retention times are slightly greater than those of the normal alcohol acetates.

tr = trace (< 0.1 %).

Table III

Composition of free primary alcohols present in the spike wax of seven barley genotypes (weight % as acetates).

Number of Carbons	Bonus	q^{42}	cq^{645}	qu^{813}	*cer-* cqu^{124}	c^{36}	cu^{108}
20		tr	tr		tr	0.1	tr
22	1.5	2.9	2.1	1.0	1.8	2.7	2.5
23	tr	tr	tr		tr	0.1	
24	6.5	8.1	7.8	5.7	6.9	7.9	7.0
25	0.5	0.6	0.5	0.3	0.4	0.8	
26	50.8	46.3	48.5	51.7	46.9	36.6	45.8
27	0.6	0.6	0.5	0.5	0.5	0.9	0.4
28	16.4	15.5	16.1	16.6	15.7	15.2	17.2
29	1.1	2.1	1.1	1.7	1.1	1.4	1.3
30	12.0	14.5	12.3	11.9	12.5	13.7	15.1
31	1.2	0.9	1.1	1.0	1.2	2.2	0.6
32	9.1	8.3	10.0	9.7	12.3	17.0	10.0
33		tr	tr		tr	tr	
34	0.2	0.3	tr	tr	0.5	1.4	tr

tr = trace (< 0.1 %)

Table IV

Composition of free acids present in the spike wax of six barley genotypes (weight % as methyl esters)[a].

Number of Carbons[b]	Bonus	q^{42}	qu^{813}	*cer-* cqu^{124}	c^{36}	cu^{108}
16	4.8	3.0	3.3	2.6	2.8	6.5
17	tr		tr	tr	tr	0.2
18	9.7[c]	1.1	4.6	1.8	3.1	4.0
19	tr	tr	0.1	tr	tr	tr
20	9.9	9.9	15.1	10.7	15.2	12.2
21	0.3	0.2	0.2	0.3	0.6	
22	14.0	15.1	16.2	15.3	20.9	14.9
23	0.5	0.7	0.4	0.5	1.2	2.8
24	12.9	18.8	12.7	16.9	19.0	11.9
25	0.3	0.4	1.1	0.7	0.5	4.1
26	9.2	15.7	10.3	15.4	11.8	11.4
27	0.2	0.8	0.8	0.3	0.2	0.7
28	16.2	16.6	13.1	16.2	12.8	15.9
29	0.7	0.8	tr	0.9	0.5	tr
30	12.0	10.4	9.7	10.4	7.1	10.2
31	0.5	tr	tr	0.7	0.1	tr
32	6.1	6.3	9.3	6.3	3.5	2.9
33	tr	tr	tr	tr	tr	tr
34	0.3	0.1	0.5	tr	0.1	tr
36	0.1	tr				
Unknowns[d]	2.1	0.1	2.5	1.0	0.4	1.3

a. Datum for *cer-*cq^{645} not available.
b. The trace to very small amounts of C_{14} often observed are not included since their volatility leads to preferential losses during isolation and preparation of the methyl esters.
c. Probably overestimated because of contamination of wax sample by internal lipids.
d. Primarily two components which elute shortly before normal C_{18} and C_{20} acid methyl esters.

tr = trace (< 0.1 %).

on a random assortment of the ester acid and alcohol moieties revealed a deficit in the amount of the observed longer chain homologues (37). The present results may still underestimate by a few percent the amount of these longer homologues. A comparison of the Bonus ester distribution given in Table V with that obtained using the Dexsil-300 column resulted in higher amounts of the longer homologues. This is expected with the latter somewhat more polar type of column packing which allows more effective separation of esters with greater than 50 carbons than does SE30 or SP-2100. A Dexsil-300 column packing has not been used in the present work, however, as we have been unable to find conditions under which excessive tailing of the peaks does not occur.

Two different types of ester are known to occur in the wax of barley spikes (37). The longer esters containing an alkan-1-ol moiety are present in all genotypes. In addition, Bonus, *cer-*c^{36} and *-*cu^{108} as well as the earlier studied *cer-*u^{69} (38) also contain shorter esters having an alkan-2-ol moiety. The most prominent esters among the former have 42, 44 and 46 carbons while among the latter they have 33 or 35 carbons. The present analyses verify the earlier report that the proportion of the esters containing alkan-2-ols is greater in *cer-*c^{36} than in Bonus and *cer-*u^{69} (35). The esters from the spike wax of *cer-*cu^{108} are characterized by having the same elevated amount of the alkan-2-ol containing esters as *cer-*c^{36}. The chain length distributions of the esters from *cer-*q^{42}, *-*cq^{645},

Table V

Composition of esters present in the spike wax of seven barley genotypes (weight %).

Number of Carbons	Bonus	*cer-* q^{42}	cq^{645}	qu^{813}	cqu^{124}	c^{36}	cu^{108}
Alkan-2-ol esters							
29	tr						tr
31	1.9					1.8	3.7
33	11.8					13.7	14.6
35	16.9					31.3	29.2
37[a]	3.7					7.4	6.7
Unknowns[b]	tr					0.1	0.4
Alkan-1-ol esters							
36		tr	tr	0.5	0.2		
37		tr	tr	tr	tr		
38	2.5	3.7	3.1	3.0	3.1	2.6	2.0
39	tr	0.2		tr	0.1	tr	tr
40	6.1	8.6	8.2	7.9	8.1	5.2	4.3
41	0.5	0.8	0.9	1.0	0.8	0.5	0.4
42	13.1	20.0	20.0	19.2	19.7	11.9	11.4
43	0.5	0.9	0.8	0.8	0.8	m	0.4
44	15.5	22.8	22.8	22.5	23.3	12.6	11.8
45	m	1.2	1.1	1.1	1.2	m	m
46	16.7	25.4	25.7	26.4	25.6	9.7	10.1
47	m	m	m	m	m	m	m
48	6.5	9.8	9.6	9.8	9.1	3.0	3.4
49	m	m	m	m	m	m	m
50	3.4	4.6	5.5	5.1	5.2	tr	1.5
51	m	m	m	m	m		
52	1.1	1.9	2.4	2.7	2.1		tr
53		m	m				
54		tr					

a. Probably contains a small amount of 36 carbon alkan-1-ol esters (see 37). On the GLC columns used, 37 carbon alkan-2-ol and 36 carbon alkan-1-ol containing esters appear as a single peak.

b. Semilog plots of apparent carbon number vs. retention time from isothermal GLC suggests that they are C_{30}, C_{32}, C_{34} and C_{36} alkan-2-ol containing esters.

tr = trace (< 0.1 %).

m = minor odd chain length alkan-1-ol containing ester integrated together with the preceding even chain homologue from which they were not completely separated by the GLC columns used. Visually they are of the same size or smaller than the shorter odd chain length homologues which were better separated by the columns.

-qu^{813} and *-cqu*^{124} are very similar. If the distribution of only the alkan-1-ol containing esters from Bonus is calculated, it appears analogous to the ester distributions in the genotypes lacking alkan-2-ol esters. When similar calculations are made for *cer-c*^{36} and *-cu*^{108} esters, however, the resulting alkan-1-ol ester distributions have relatively more of the shorter homologues C_{38}, C_{40} and C_{42} and less of the longer ones C_{46}, C_{48}, C_{50} and C_{52}.

Table VI presents the chain length distributions of the acid moieties (analyzed as their methyl ester derivatives) from each of the ester samples studied in Table V. They are remarkably

Table VI

Composition of ester acids from the spike wax of seven barley genotypes (weight % as methyl esters).

Number of Carbons	Bonus	*cer-* q^{42}	cq^{645}	qu^{813}	cqu^{124}	c^{36}	cu^{108}
14						tr	
15						tr	
16	11.0	7.2	6.7	8.0	3.4	6.0	4.3
17			tr	tr	tr	tr	
18	12.3	10.4	8.7	16.4	9.1	14.8	18.0
19	0.1	tr	tr	0.2	tr	0.3	0.1
20	57.1	54.0	51.2	54.8	57.2	56.8	59.3
21	tr	0.7	0.9	0.6	1.0	1.0	0.5
22	16.5	20.7	21.1	15.9	20.8	18.5	16.3
23	tr	0.3	tr	tr	tr	0.1	tr
24	2.9	5.6	9.1	2.8	6.5	2.1	1.7
25	tr	tr	tr		tr	tr	
26	0.1	1.1	2.1		1.9	0.3	tr
27					tr		
28			0.1		0.1		
Unknowns[a]		tr	0.1	1.3	tr	0.1	

a. Consists of two components which have slightly shorter retention times than normal C_{18} and C_{20} acid methyl esters.

similar in composition to one another and also to the ester acids from *cer-*u^{69} spike wax (38). The spectrum of the ester acids differs from that of the free acids in consisting primarily of C_{22} and shorter chain lengths. Among these the C_{20} homologue predominates, varying from 51 to 59% by weight of the ester acids.

The composition of the alcohol moieties (analyzed as their acetate derivatives) from each of the ester samples studied in Table V is given in Table VII. Both alkan-2-ols as well as the omnipresent alkan-1-ols occur in the Bonus, *cer-*c^{36} and *-*cu^{108} spectra as also reported for *cer-*u^{69} ester alcohols (38). As expected from the relative amounts of the two ester types (Table V) and the similarity of the ester acid distributions (Table VI), the amount of the short alcohols is greater in *cer-*c^{36} and *-*cu^{108} than in Bonus (Table VII). Since the alkan-2-ols, especially C_{13}, are quite volatile compared to the longer alkan-1-ols, the data given underestimate the actual percent by weight of the alkan-2-ols (see 37). The chain length distributions of the alkan-1-ols from the esters of the genotypes *cer-*q^{42}, *-*cq^{645}, *-*qu^{813} and *-*cqu^{124} are essentially indistinguishable with the C_{22}, C_{24} and C_{26} chain lengths accounting for 76 to 86% of the total. The spectra of the four genotypes can be compared with those obtained by calculating the distributions of only the alkan-1-ols in *cer-*u^{69} (38), Bonus, *cer-*c^{36} and *-*cu^{108}. When this is done the chain length distributions for Bonus (C_{22}, 21.7%; C_{24}, 23.6%; C_{26}, 36.8%) and *cer-*u^{69} (C_{22}, 30.4%; C_{24}, 28.5%; C_{26}, 35.3%) are very like those for the four alkan-1-ol ester only containing genotypes. The calculated spectra for *cer-*c^{36} and *-*cu^{108} differ slightly by having somewhat more of the C_{22} homologue (35.4 and 35.2%, respectively) and less of the C_{26} homologue (23.4 and 29.9%, respectively). This is in agreement with the somewhat different alkan-1-ol containing ester distributions for these two genotypes mentioned above.

3.3. Origin of *cer-c*, *-q* and *-u* mutants

The number and type of mutational events induced by various agents at the *cer-cqu* region are summarized in Table VIII. The 434 mutational events included represent 34.7% of the

Table VII

Composition of the ester alcohols from the spike wax of seven barley genotypes (weight % as acetates).

Number of Carbons	Bonus	*cer-q*^{42}	*cer-cq*^{645}	*cer-qu*^{813}	*cer-cqu*^{124}	*cer-c*^{36}	*cer-cu*^{108}
Alkan-2-ols							
13[a]	4.0					3.4	3.5
15	7.8					35.8	48.0
17[b]	0.6					0.6	1.3
Alkan-1-ols							
14		tr		tr			
15		tr		tr			
16		1.0	0.2	6.3	1.0		
17		tr		0.1	tr	tr	tr
18	0.3	1.3	0.2	6.5	3.1	0.4	0.6
19	tr	tr		0.5	tr	tr	tr
20	1.8	3.1	2.3	3.0	2.7	2.1	2.0
21[c]	tr	tr		tr	tr	tr	tr
22	19.0	29.6	23.6	24.9	26.9	21.3	16.6
23	0.4	0.4	0.2	0.2	0.4	0.3	tr
24	20.7	24.8	23.1	21.7	24.3	15.9	11.1
25	0.5	0.5	0.5	0.5	0.6	0.3	0.1
26	32.3	31.3	38.1	29.6	32.3	14.1	14.1
27	0.2	0.2	0.2	0.4	0.4	tr	tr
28	5.8	4.4	6.9	4.8	5.3	1.8	2.1
29	0.2	0.8	0.2	0.3	0.4	0.2	tr
30	2.1	1.3	2.3	0.8	1.6	0.9	0.5
31	0.3	0.2	0.3	tr	0.1	0.1	tr
32	4.1	1.2	1.8	0.3	0.9	2.8	tr
33		tr				tr	
34		tr				tr	

a. Values are underestimated. The volatility of this alcohol leads to preferential losses during preparation of the alcohol acetates (see 37).
b. Includes C_{16} alkan-1-ol which is not separated from a C_{17} alkan-2-ol under the GLC conditions used.
c. Presence of C_{21} is assumed since both C_{19} and C_{23} are found. The amount is not measurable as contamination by a phthalate having the same retention time occured during sample preparation. (see section 2.1).

tr = trace (< 0.1 %)

mutations that have been assigned to all *cer* loci to date. The data confirm the earlier drawn conclusion that *cer-c*, *-q* and *-u* are most easily mutated by sulfonates (22). Ethyleneimine is the second most useful agent for inducing mutations in this region. Neutrons, γ-rays and X-rays, by comparison, are much less efficient. The frequency of multiple events in the *cer-cqu* region is relatively high being 3 %. Interestingly, only one of the 13 multiple events was induced by ethyl methane sulfonate, whereas 11 were induced with neutrons and one with γ-rays.

3.4. Phenotypic observations

The *cer* mutants have been grouped according to their effect on the apparent amount of wax present on the spikes, the uppermost internodes plus leaf sheaths and the leaf blades. The amount of wax on each organ of the wild type is given as ++, a reduced amount as + and a lack of wax as a − (16). The normal phenotypic formula for *cer-c* and *-q* mutants is − − ++ and for *cer-u* mutants it is + + ++. During the past two to fifteen years, however, some of the mutants have had variable spike phenotypes in field observa-

Table VIII

Origin of the single and multiple mutational events at the *cer-cqu* region[a].

Mutagenic agent	Number of events involving *cer-*						
	c	*q*	*u*	*c* + *u*	*q* + *u*	*c* + *q*	*c* + *q* + *u*
Neutrons	8	5	2	2	2	1	6
γ-rays	8	5	5				1
X-rays	4	7	1				
Ethyl methane sulfonate	61	39	53	1			
Other sulfonates	24	17	20				
Ethyleneimine	28	21	24				
Other	31	31	27				
Total	164	125	132	3	2	1	7

[a] Data from references 16, 21 and 22.

tions (21). More explicitly, among the 164 alleles of *cer-c*, 148 were always −, but 16 were either + or −. Among the 125 alleles of *cer-q*, 109 were always −, while the remaining 16 were either + or −. Of the 125 alleles of *cer-u*, 55 were always +, whereas the other 80 were either + or −. *Cer-c*^{3}, *-c*^{36}, *-q*^{35} and *-q*^{42} were among those that were consistently classified as lacking wax on their spikes as were all the multiple mutants. *Cer-u*^{69} was always recorded as +, whereas *cer-u*^{58} was given a + in 14 years and a − in one year.

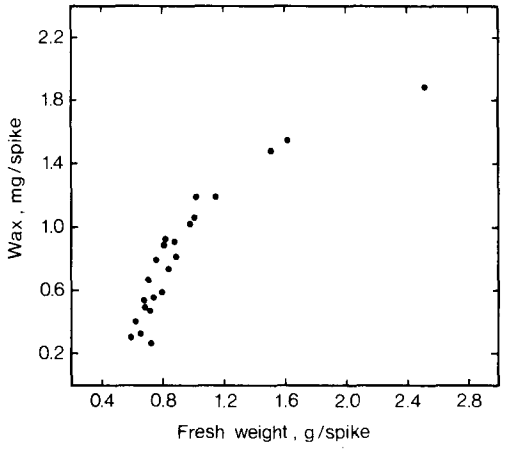

Figure 2. Relationship between the amount of wax and the fresh weight of Bonus spikes during the two to three weeks following their emergence from their leaf sheaths.

Regardless of where the three F_1 types, produced by making pairwise crosses among *cer-c*^{3}, *-q*^{35} and *-u*^{58}, were grown, they could be distinguished from wild type plants at the stage when the spikes were almost or just completely emerged from their leaf sheaths. At this time they look yellow green, that is appearing to lack wax. By the time the spikes of the F_1 types are fully developed, however, their spikes are as blue grey as the wild type, that is appearing to possess a full amount of wax. This observation presumably explains why all of the *cer-q* mutants, at least, were earlier described as being semi-dominant (22). In this respect the relationship between the increase in fresh weight of a wild type Bonus spike and the amount of wax present on that spike is of interest, and is shown in Figure 2. The amount of wax present when the spikes are emerging (0.2 to 0.6 mg) increases at least two-fold in the succeeding two to three weeks. Under optimal environmental conditions spike maturation takes a minimum of four to five weeks from the time of their emergence (4). At what time wax synthesis stops has not been ascertained, although the rate has decreased considerably by the end of the two to three weeks (Figure 2). Combining these observations one is led to hypothesize that heterozygotes for mutants of *cer-c*, *-q* and *-u* synthesize wax, especially β-diketones, more slowly than the wild type.

Allele tests between *cer-cqu*^{420} and each of the mutants *cer-c*^{3}, *-q*^{35} and *-u*^{58} gave rise to

progeny with an *eceriferum* phenotype, confirming earlier results (22).

3.5. Test cross analyses

The results of the three test crosses demonstrate that *cer-c*, *-q* and *-u* are remarkably close together. That is, in the test cross $c + +/+ + u \times cqu/cqu$ no wild types were found among the 8096 progeny. The presence of a single wild type arising via a recombinational event in a test cross progeny of this size would be equivalent to a recombination frequency of 2.5×10^{-4}; 0.025 map units. Thus, the conclusion can be drawn that *cer-c*^{3} and *-u*^{58} are closer together than 0.025 map units. Similarly, the lack of wild type plants in the 9361 progeny of the $+q +/+ + u \times cqu/cqu$ test cross indicates that *cer-q*^{35} and *-u*^{58} are closer together than 0.021 map units. The lack of wild type progeny among the 9476 individuals of the $c + +/+q + \times cqu/cqu$ test cross infers that *cer-c*^{3} and *-q*^{35} are also closer together than 0.021 map units. Since in all three test crosses we failed to recover any recombinant gametes, it is not possible to determine the linear arrangement of *cer-c*, *-q* and *-u* along the chromosome.

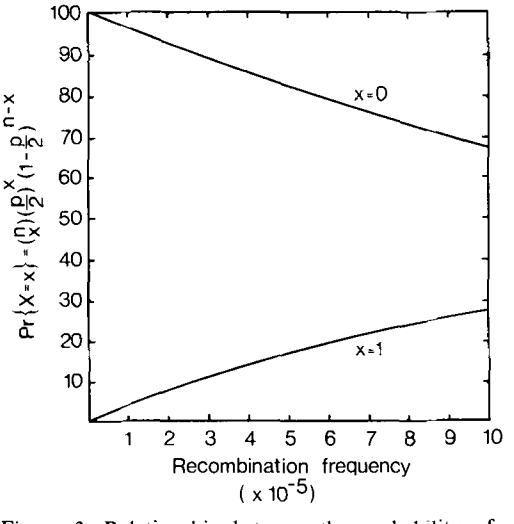

Figure 3. Relationship between the probability of finding none (upper, Pr {X = 0}) or one (lower, Pr {X = 1}) wild type recombinant, which has a probability of $\frac{p}{2}$ of occurring in a progeny of 8096 (n) individuals if the two markers *cer-c*^{3} and *-u*^{58} recombine with various frequencies (see section 2.2. and equation [1].

If *cer-c*^{3} and *-u*^{58} are less than 0.025 map units apart, the question can be asked how close they are likely to be. Figure 3 illustrates the relationship between the recombination frequency and the probability of actually observing one or no recombinational events in a test cross progeny of 8096 plants. In the upper curve the number of recombinational events is set equal to zero, that is, what we found. Such an experimental result is expected 95% of the time if the two markers *cer-c*^{3} and *-u*^{58} recombine with a frequency of 1.27×10^{-5}, that is, they are 0.0013 map units apart. Similar analyses indicate that no recombinational events will be found 95% of the time when *cer-q*^{35} and *-u*^{58} are 0.0011 map units apart and when *cer-c*^{3} and *-q*^{35} are also 0.0011 map units apart.

Since we have calculated above that *cer-c*^{3} and *-u*^{58} must be closer together than 0.025 map units, it is of interest to know what the probability is of finding the one recombinational event upon which this prediction is based. That the probability is quite low when the recombination frequency is less than 1×10^{-4} is illustrated by the lower curve in Figure 3 in which the expected number of recombinational events is set equal to one. If the horizontal axis is extended and the lower curve continued to the recombination frequency of 2.5×10^{-4} (0.025 map units), the maximum probability of finding the one recombinational event is 36.8%. Similar analyses can be made for the other two test crosses with the same results of a maximum probability of 36.8%.

If the data from the three test crosses are pooled, that is, no recombinational events among 26,933 gametes, an estimate of the map units between the most distant of the three markers can be obtained using equation [3]. This analysis is presented in Figure 4. The upper curve, as in Figure 3, sets the expected number of recombinational events equal to zero which is the observed result. It is expected 95% of the time if the distance between the two markers farthest apart is 0.0003 map units. When the map distance is so small and the progeny size is so large, the probability of not finding any recombinational events decreases quite rapidly as the recombination frequency increases from zero to

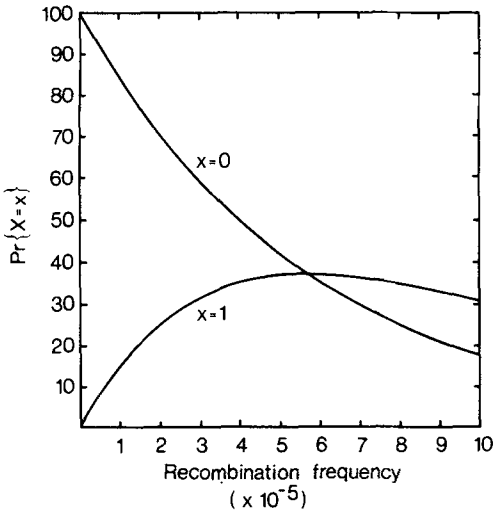

Fig. 4. Relationship between the probability of finding none (upper, Pr {X = 0}) or one (lower, Pr {X = 1}) wild type recombinant in the pooled test cross progeny of 26,933 individuals, if the three markers *cer-c*[3], *-q*[35] and *-u*[58] are arranged in a linear sequence with the two distances being equal. The probability of finding the wild type recombinant equals $\frac{p}{2}$ for each of the two shorter distances and equals p for the longer distance (see section 2.2. and equation [3]).

1×10^{-4}. Compare upper curves in Figures 3 and 4.

4. DISCUSSION

In the de novo synthesis of fatty acids, a number of short carbon chains are successively donated by an activated elongating unit to an activated primer until a given chain length, most frequently C_{16}, is attained. Each individual addition requires a number of distinct reactions carried out by enzymes such as β-ketoacyl synthetase, β-ketoacyl reductase, β-hydroxyacyl dehydrase, β-enoyl reductase and various transacylases. In many instances these enzymes are soluble whereas in some cases they are membrane bound. With regard to their molecular organization, the soluble fatty acid synthetase systems may be aggregated into a multienzyme complex as in yeast, or they may be non-aggregated as in *E. coli* or in spinach chloroplasts. Numerous modifications in all facets of this basic process have been described which are responsible for the variety of the end product(s). Although considerably less is known about elongation beyond lengths of 16 carbons, as must occur in wax biosynthesis, presumably an analogous set of enzyme reactions is required at each elongation step. Epicuticular wax biosynthesis which is carried out by epidermal cells (2, 14) consists not only of the elongation steps but also of the associated pathways giving rise to the various lipid classes. Thereafter the wax is deposited on the cuticle surfaces. Presently available experimental evidence suggests that the site of wax biosynthesis is the plasmalemma (see 36), although it is not possible to exclude that some or all of the reactions actually take place in the outermost cell wall.

The results of the chemical analyses of the wax composition on the spikes of the wild type and the six *cer* mutants together with that for *cer-u*[69] (38) confirm the sites of actions of *cer-c*, *-q* and *-u* indicated in Figure 1. The effect on the β-diketones, hydroxy-β-diketones and alkan-2-ol containing esters in the four multiple mutants was always that of the gene controlling the earliest step in the biosynthetic sequence. The overall independence of this pathway (Figure 1) from those involved in the synthesis of the other wax classes is demonstrated by the remarkable similarity in the composition of these other wax classes in the wild type and these *cer* mutants. Small differences in the chain length distributions were found among the aldehyde and primary alcohol distributions. Since both of these lipid classes are relatively minor constituents of the spike wax, it is not possible to say whether they indeed reflect significant alterations in the wax biosynthesis. A small modification was also noted for alkan-1-ol containing ester chain length distributions from *cer-c*[36] and *-cu*[108] attributable to a decrease of the C_{26} and increase of the C_{22} alcohol moieties. This is the only indication that the pathway shown in Figure 1 may not be completely independent from those leading to the other wax classes.

Numerous proteins have more than one functional domain, that is, catalytic or binding site. In recent years, a number of these proteins have been shown to be a single polypeptide chain or a multimer formed by aggregation of one or more sets of identical polypeptide chains (see 12).

If at the protein level, a gene is considered to determine a single polypeptide chain, then it follows that a single gene can control more than one function. Such genes are referred to as multifunctional genes herein, although they have also been called cluster genes (7, 36). One of the best studied examples of this phenomenon from both the genetic and biochemical aspects is the soluble fatty acid synthetase complex of yeast (see 27). In fact, two such multifunctional genes, *fas*–1 and *fas*–2, are involved. Both genes code for multifunctional polypeptides having at least three functional domains each. The active *in vivo* yeast fatty acid synthetase is an aggregate of 6 α plus 6 β polypeptide chains, where α is the product of *fas*–2, and β the product of *fas*–1.

While genes controlling enzymes in a given biochemical pathway are generally dispersed throughout the genome in eukaryotes, many examples are known in which two or more of the enzymes in the same pathway but not necessarily concerned with sequential reactions are determined by a cluster of closely linked genes. In the past few years such apparent gene clusters have been shown in a number of cases to be in fact multifunctional genes (e.g., 1, 6, 7, 27). The question can be asked whether the *cer-cqu* region in barley represents such a multifunctional gene or is really a cluster of individual genes.

While the genetic tests failed to reveal the linear order of the investigated mutants, they did demonstrate that the farthest apart any two of them could be was 0.025 map units. The best estimate of the actual distance was 0.0011 to 0.0013 map units apart. This close proximity suggests that perhaps the *cer-cqu* region represents a single locus. Three recent preliminary reports of attempts to construct maps of single loci in barley gave the following results. Four mutants at the *li* locus gave recombination frequencies up to approximately 3×10^{-4}; 0.03 map units (13). Six alleles at the *ml-o* locus were mapped to three sites, the outer two of which recombined with a frequency of approximately 4×10^{-4}; 0.04 map units (11). Seven mutants at the *glx* locus gave recombination frequencies ranging from approximately 1 to 200×10^{-5}; 0.001 to 0.2 map units (26). By comparison a recent map of 19 genes on chromosome 5 in barley, constructed using a maximum likelihood method to estimate gene distances, shows the two closest loci to be 0.6 map units apart (10). The 18 of the remaining identified 67 *cer* loci that have been mapped thus far appear to be randomly distributed throughout the genome (5, 9, 21, 29, 30). Where measured, none of these *cer* loci are closer together than one map unit. The most tightly linked are *cer-i* and *-zj* which are 1.1 map units apart (30). In conclusion, the presently available genetic plus biochemical data suggest that the *cer-cqu* region is a multifunctional gene. Should this be true, then the wild type phenotype of the fully developed F_1 spikes seen in crosses such as $c \times q$, for example, would indicate that the polypeptide product of this gene formed a functional aggregate consisting of at least two chains. Whether or not the *cer-q* phenotype, for example, is always the result of a defect in the same domain or whether it encompasses several different domains involved in the elongation system as the *fas*–1 and *fas*–2 products do will have to be determined.

The 13 multiple mutational events in the *cer-cqu* region can conceivably have arisen in one or more of the following ways. Some of them may be deletions. The finding of all possible double combinations, however, eliminates the possibility that all of them are deletions. This same argument excludes the possibility that they all determine nonsense or frameshift mutations in a single multifunctional gene. Alternatively, they may be due to concurrent single point mutations. This suggestion (22) follows from the observation that 11 of the 13 multiple events are neutron induced. It has previously been predicted that neutrons, being a more densely ionizing radiation than say X-rays, would have a greater probability than X-rays or chemical mutagens of causing more than one mutation by a single primary event (15). On the other hand, this hypothesis is not supported by the study of reverse mutations within the first 33 nucleotides of the *cyl* gene in yeast (28). The data in the latter study allow X-rays to be compared with α-rays that are even more densely ionizing than neutrons. To specify, among 52 reversions of the mutant *cyl-179*, 48 were found to be due to single and four to multiple base changes. X-rays accounted for eight of the single and two of the multiple events, whereas α-rays accounted for nine of the single and one of the multiple events. Only single base changes were identified when

the 53 reversions of *cyl-183* were studied. Twelve of these were induced by X-rays and six by α-rays. Extrapolation from this data suggests that some of the apparent multiple mutational events in the *cer-cqu* region are the result of single mutational alterations. Given that the *cer-cqu* region represents, as suggested above, a multifunctional gene, then a single mutation could cause a modification of the polypeptide chain which knocks out more than one functional domain. The *cer-c*, *-q* and *-u* mutations which each block synthesis at a single step, as shown in the pathways of Figure 1, would then be caused by alterations affecting only a single domain of the multifunctional polypeptide.

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