GENETIC FINE STRUCTURE ANALYSIS OF THE *AMYLOSE-EXTENDER* LOCUS IN *ZEA MAYS* L.[1]

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ABSTRACT

On the basis of interallelic recombination frequencies measured in diallelic crosses of the 5 *amylose-extender* alleles in maize, *ae*, *B1*, *B3*, *M2* and *i1*, it was possible to construct a unique linear genetic map ordering all 5 alleles within the locus. The reciprocal diallelic crosses each gave comparable frequency estimates. The relative order is *ae*, *i1*, *B3*, *B1*, *M2* or the reverse. Even though F1 endosperms resulting from all possible diallelic crosses were phenotypically mutant, therefore non-complementing, no decision as to whether or not these alleles exhibit functional complementation should be made without biochemical characterization of the starches from the various heteroallelic genotypes.

THE occurrence of intragenic recombination in at least two higher organisms has recently been confirmed. NELSON (1957) devised an efficient scoring system in maize whereby *waxy* (*wx*) mutant and revertant or recombinant normal (*Wx*) genotypes could be distinguished in preparations of pollen grains on the basis of differential iodine staining properties. Following the measurements of *Wx* frequencies in numerous *wx* mutant strains as well as crosses among them, 24 non-complementing alleles could be ordered within the locus on the basis of frequencies of *Wx* gametes measured in both pollen (NELSON 1959, 1968) and conventional (NELSON 1962) analyses. Apparently the *wx* cistron is associated with a single enzyme, starch granule-bound glucosyl transferase (NELSON and RINES 1962; NELSON and TSAI 1964), but it is not clear whether *wx* is a structural or regulatory gene for this enzyme.

In addition to other pollen analyses with *wx* alleles (BRIGGS and SMITH 1965; BRIGGS 1968; BIANCHI and CONTIN 1963) interallelic recombination has also been demonstrated at the *glossy-1* (SALAMINI and BORGHI 1966), *shrunken-2* (NELSON 1969), and *sugary*-1 (SALAMINI 1967) loci by conventional procedures.

The best indication that intragenic recombination occurs in Drosophila was

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obtained utilizing ingenious selective procedures by CHOVNICK and his collaborators (1962, 1964, 1970) who demonstrated recombination among several rosy (ry) mutants. No allelic complementation has been detected among these mutants, which completely lack xanthine dehydrogenase activity (SCHALET, KERNAGHAN and CHOVNICK 1964). GRELL (1962) and YEN and GLASSMAN (1965) presented evidence that *ry* is the structural gene for this enzyme.

Interallelic recombination has also been demonstrated for *Notch* (WELSHONS 1965), *rudimentary* (GREEN 1963) and *maroon-like* (CHOVNICK *et al.* 1969; FINNERTY, DUCK and CHOVNICK 1970), but each has been interpreted as being a single locus exhibiting some allelic complementation. Garnet mutants (HEXTER 1958; CHOVNICK 1961), as well as miniature and dusky (DORN and BURDICK 1962), and white mutants (GREEN 1959; JUDD 1959; STERN 1969) also seem to map within a single functional unit, although these loci generally have been considered complex. Recently, GREEN (1969) mapped a controlling element which he concluded is integrated at the *white* locus and reduces interallelic crossing over among white mutants.

The genetic fine structure analysis of a second locus in maize which utilizes the pollen-scoring system, *amylose-extender* (*ae*), is presented in this paper. It was made possible by the fact that while normal (*Ae*) and mutant (*ae*) pollen starches both contain amylose and are indistinguishable when iodine-stained, red-staining *Ae wx* and dark-staining *ae wx* may be differentiated due to the presence of amylose in pollen starch of only the latter genotype. The *ae* gene alone is associated with the production of high amounts of amylose without greatly reducing total amounts of starch (FERGASON, HELM and ZUBER 1966). On the basis of interallelic recombination frequencies measured in diallelic crosses of 5 *ae* mutants, a linear genetic map of the locus is proposed.

MATERIALS AND METHODS

Mutant alleles: The standard allele is *ae*, the one in general use by maize geneticists, and was obtained from Dr. H. H. KRAMER, Purdue University. The other 4 alleles have been provided with tentative symbols for experimental purposes and ease of presentation. Mr. R. P. BEAR of the Bear Hybrid Corn Company, Decatur, Illinois, provided ae^{B1} (Bear-1) and ae^{B3} (Bear-3). Dr. M. Z. ZUBER, University of Missouri, provided ae^{M2} (Missouri-2). The ae^{i1} mutation arose in a stock grown at Purdue University known to be carrying the activator-dissociator (*Ac-Ds*) system of gene-controlling elements described by MCCLINTOCK (1965a, 1965b); thus, the designation induced-1 (*i1*) was assigned. Hereafter, these alleles will be denoted by their superscripts *B1*, *B3*, *M2* and *i1*, respectively, and only the standard will be specificied *ae*.

The waxy allele used in this study was the standard *wx*.

Inbreeding: The *ae* and *wx* mutants were incorporated into the inbred line L317 in a backcross program at The Pennsylvania State University. All *ae* alleles were not backcrossed the same number of times to L317 (*ae* BC-3; *B1* BC-2; *B3* BC-2; *M2* BC-1; and *i1* BC-1) since they were made aavilable at different times. It was desired that each stock be as nearly isogenic as possible to minimize background effects in the analysis; therefore, these 5 lines possessing the greatest number of backcrosses among the several available *ae* lines were selected.

Pollen analyses: doubly mutant homo- and heteroallelic plants were greenhouse grown during the spring of 1969. Their central spikes were removed early on days they expected to shed pollen. Tassels were rinsed with tap water to remove foreign pollen and stored in 75% ethyl alcohol for at least one month. This "curing" period was essential for pollen to retain the iodine stain.

The pollen staining procedure was modified from the one CREECH and KRAMER described (1961). The staining solution was prepared by dissolving 30 g granular KI in 40 ml 25% dimethyl sulfoxide (DMSO); 6.5 g I_2 were then added before diluting the mixture with 60 ml 25% DMSO. Pollen was strained in the following manner: Twenty-seven to thirty-six anthers were removed from proximal florets and placed in a micro homogenizing flask with 1 ml of the iodine solution. Anthers were allowed to stand 2 min, homogenized for 30 sec with a VirTis "23" homogenizer, then permitted to stand 4.5 min. The suspension was filtered through Curity Grade 90 cheesecloth onto filter paper in a suction funnel. The flask and anthers were rinsed with 8 ml distilled H_2O. After removing the staining solution by suction, 2 ml of 25% ethanol were added to the grains and allowed to remain for 30 sec. This procedure, unlike NELSON's (1968), overstains all pollen in order that the 2 starch types of *ae wx* containing only about 10% amylose and *Ae wx* with no amylose can be reliably distinguished. In preliminary staining tests, it had been clear that some *ae wx* pollen failed to stain sufficiently to be unequivocally differentiated from *Ae wx*; thus the need for designing a method of overstaining, with controlled destaining during a subsequent step.

The stained grains were washed from the filter paper into a watch glass with distilled H_2O. Following removal of the H_2O by vacuum, 0.7 ml of a gelatin medium, consisting of 0.6 g gelatin, 30 ml H_2O and 3 drops Tween 80, was added to the pollen. The gel suspension was poured onto an 80 × 100 mm glass slide, evenly spread, and covered with a 40 × 60 mm cover glass. Once the mixture gelled, edges of the slip were sealed with colorless fingernail polish and preparations were stored at 8°C.

The slides were examined after 18 hr with a Leitz stereo-scopic microscope with incident and transmitted light. The position of each *Ae wx* red-staining pollen grain, if any at this time, was marked on the cover slip with a red nylon tip marker. Each slide was then heated at 54°C for 2 min and scored again. This step was repeated 3 times if necessary, or until red grains were unequivocally differentiated. Unfortunately, since pollen is killed during this procedure, one cannot verify presumed *Ae wx* types as with the kernel phenotypic procedures.

The total population of pollen grains on each slide was estimated from actual counts of 12 randomly preselected areas on the slide. Each sample area of 28.26 mm^2 was photographed using a Leica 35 mm camera fitted on the microscope with the MIKAS microattachment plus an 1/3× intermediate adapter. The developed film was projected onto a grid screen and the grains were counted. The total number of pollen grains in the 12 sampling areas was multiplied by the constant 8.488, the number of equivalent areas on a slide, to obtain an estimate of the total number of grains per slide.

RESULTS AND DISCUSSION

The frequencies of red-stained pollen grains from plants produced following diallelic crossing of strains having the 5 *ae* alleles are listed in Table 1. Comparable recombinational estimates were obtained in each of 2 reciprocal crosses analyzed, *B1* × *B3* and *B3* × *B1* as well as *B1* × *M2* and *M2* × *B1*; in the latter, the *M2* male and female parent, respectively, was the same plant. A third reciprocal cross, *ae* × *M2* and *M2* × *ae*, was sampled later in the year and also resulted in very similar estimates (Table 2).

When the 5 *ae* alleles were crossed in all possible combinations the phenotypes of F_1 kernels of diallelic constitutions could not be distinguished from those of homoallelic seeds. All possessed the translucent, semi-collapsed property characteristic of the *ae ae ae wx wx wx* genotype. From this standpoint, the 5 alleles did not exhibit complementation.

The incidences of presumed *Ae wx* types in homoallelic lines are shown in Table 3. They are higher in every set of heteroallelic crosses, ranging from 12.2 to 87.4 × 10^{-5}, than in homoallelic lines, which range from 2.3 to 3.3 × 10^{-5}. It

TABLE 1

Frequencies of Ae wx *pollen from heteroallelic plants**

Crosses of *ae* alleles (all *wx wx*)	Estimated total number of pollen grains sampled	Total number of *Ae* observed	Individual plant frequencies of *Ae*	Mean frequency of $Ae \times 10^{-5}$ $\pm S_{\bar{x}}$
B1 × *B3*	474,334	59	11.5, 8.2, 14.8, 14.3	12.2 ± 1.5
B3 × *B1*	354,297	49	15.9, 10.2, 16.1, 13.9	14.0 ± 1.4
i1 × *B1*	1,145,455	273	33.1, 48.1, 7.3, 10.8, 15.5, 19.3, 21.3, 50.6, 30.1, 52.3, 22.9, 34.6	28.9 ± 4.5
i1 × *M2*	683,887	486	80.3, 30.3, 83.9, 79.4, 76.6, 64.9, 66.8	68.9 ± 7.0
B1 × *M2*	339,944	72	21.9, 27.3, 15.1	21.4 ± 3.5
M2 × *B1*	974,396	200	17.0, 23.8, 25.1, 10.4, 15.5, 12.1, 33.7, 24.8, 22.0, 26.2	21.1 ± 2.3
B3 × *M2*	1,534,537	399	21.8, 11.2, 18.3, 20.5, 27.2, 36.2, 25.6, 31.3, 19.4, 13.3, 24.5, 47.1, 29.2, 21.4, 14.9, 34.9	24.8 ± 2.3
B1 × *ae*	572,345	499	82.0, 122.0, 106.5, 91.7, 96.5, 57.5, 97.1, 93.9, 39.5	87.4 ± 8.4
ae × *B3*†	234,550	97	42.0†, 39.1†, 42.5	41.2 ± 1.1
i1 × *ae*	1,323,449	721	97.8, 68.4, 30.3, 57.0, 54.6, 75.0, 77.9, 78.8, 61.1, 14.8, 27.8, 18.1, 156.0, 49.8, 63.7, 23.2, 14.4, 9.0, 18.3	52.4 ± 8.4
i1 × *B3*	984,574	190	10.3, 24.5, 14.8, 9.8, 15.9, 29.9, 17.2, 20.4, 31.2, 10.9, 11.7, 15.4, 25.2	18.3 ± 2.1

* All plants were grown in the greenhouse during late winter and spring, 1969.
† The noted two plants were segregating *Wx wx*; thus, the number of reds scored was doubled before calculating the *Ae* frequencies.

seems reasonable, therefore, to assume that the increase over background frequencies comprising reversion and/or suppressor mutations was due to interallelic recombination.

In contrast to *B1*, *B3*, *i1* and *M2*, *ae* pollen exhibited the most variable phenotype, contrasting grains within single samples often stained various shades of orange and tan. Since *ae* also exhibited this peculiar staining in samples from different tassels collected later in the year and in samples from another inbred,

TABLE 2

Frequencies of Ae wx *pollen from plants of the reciprocal crosses* ae wx × ae^{M2} wx *and* ae^{M2} wx × ae wx*

Cross (all *wx wx*)	Estimated total number of pollen grains sampled	Total number of *Ae* observed	Individual plant frequencies of *Ae*	Mean frequency of $Ae \times 10^{-5}$ $\pm S_{\bar{x}}$
ae × *M2*	671,588	419	57.2, 90.9, 52.9, 64.1, 57.1, 40.0	60.4 ± 6.9
M2 × *ae*	363,626	218	73.6, 54.4, 51.4	59.8 ± 6.9

* These plants were grown in the greenhouse during the summer and early fall, 1969.

TABLE 3

Frequencies of Ae wx *pollen from homoallelic plants**

Plant genotype (all *wx wx*)	Estimated total number of pollen grains sampled	Total number of *Ae* observed	Individual plant frequencies of *Ae*	Mean frequency of $Ae \times 10^{-5} \pm S_{\bar{x}}$
ae ae	524,346	21	0.0, 14.2, 2.8, 2.1, 3.1, 0.0, 3.5, 1.1	3.3 ± 1.6
B1 B1	410,802	13	4.1, 3.4, 2.5, 3.0, 1.6, 4.4	3.2 ± 0.4
B3 B3	800,419	20	1.8, 0.0, 2.8, 3.2, 2.3, 4.1, 3.2, 2.2	2.4 ± 0.4
i1 i1	1,167,958	28	1.6, 1.9, 1.2, 2.2, 0.0, 1.4, 0.0, 0.0, 2.4, 2.5, 4.0, 1.3, 13.0, 0.0, 3.8	2.3 ± 0.8
M2 M2	667,589	18	2.6, 2.6, 3.6, 3.8, 2.0, 1.5	2.7 ± 0.4

* All were grown in the greenhouse during late winter and spring, 1969.

the pattern may be characteristic of this allele, perhaps resulting from the presence of short amylose chains of varying lengths.

The main purpose for measuring interallelic recombination was to determine whether or not a consistent genetic map of the 5 *ae* mutants could be deduced from the recombinational data. Thus, the mean frequencies in Table 1 were reduced by the average of the respective parental mean frequencies listed in Table 3 to obtain more accurate estimates of frequencies of recombination among the mutants. The map of the *ae* locus derived from these data is shown in Figure 1

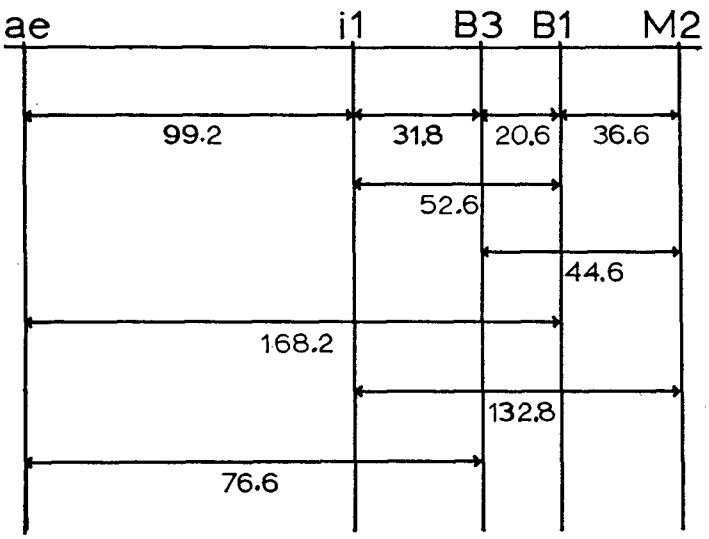

FIGURE 1.—Proposed map of five *ae* mutations, according to the data presented in the text. Map distances are mean frequencies ($\times 10^{-5}$) of *Ae wx* types in diallelic crosses (Table 1) reduced by the average of the respective parental mean frequencies (Table 3), and doubled to account for reciprocal recombinant types. The *ae–B3* value was obtained somewhat differently, as discussed in the text.

and is the only one which satisfies the data. No other array results in greater additivity.

It should be noted that *ae M2* and *M2 ae* plants were grown and sampled at a different time and under different environmental conditions than the remaining plants; therefore, they were not considered for the purpose of map construction.

B1 and *ae* recombined more often than any of the other alleles. *Induced*-1 recombined with *ae* at a greater frequency than with *B1*, the sum being only a little less than the *B1 ae* interval; thus, the order *ae i1 B1* (or *B1 i1 ae*) was defined. *B3* combined more often with *i1* than *B1*, and the sum of these 2 estimates is about equal to the *i1 B1* segment, positioning *B3* between *i1* and *B1*. Finally, *M2* combined with *i1*, *B3* and *B1* in decreasing frequencies, indicating its location is most likely at the opposite end of the locus from *ae*. Without outside marker genes the relative orders *ae i1 B3 B1 M2* and *M2 B1 B3 i1 ae* on the standard maize map cannot be distinguished.

The results of the *ae* × *B3* cross are not consistent with the proposed order. When 2 of the 3 plants analyzed were found to be segregating *Wx* vs. *wx*, the number of reds scored among *ae wx* pollen from the two was doubled to account for the estimated *ae Wx* class before calculating the estimated frequency of *Ae wx* types. The 2 *ae* classes were rather clearly distinguished on the basis of both size and staining properties; the double mutant was generally smaller and destained consistently far more rapidly than did the *ae Wx* class. This procedure may be invalid for comparison with other crosses of this study, however, if *ae wx* had a reduced transmission or viability frequency compared to *ae Wx*.

Of interest is the relative position of standard *ae* at one of the extremities of the locus and the relatively extended region separating it and the remainder of the mutants. This could suggest some regulatory or operator function sometimes associated with a site at the beginning of a cistron. On the other hand, *ae*, could be a deficiency over an extended region, but without the localization of other alleles in this region which fail to combine with *ae* this cannot be confirmed. As mentioned previously, *ae* pollen exhibits an exceptional staining pattern, perhaps the extreme expression of the phenotype.

The 5 mutations appear to occupy different positions of the *ae* locus, as evidenced by the unique genetic map constructed from these data. While all 5 of these alleles recombined, analysis of additional *ae* mutations should further define the *ae i1* segments and may reveal deficiencies, in which case recombination could not be detected in diallelic crosses involving a segment defined by a deletion mutation.

While the additivity of some distances within the *ae* locus is fairly good, it is not ideal. This feature, along with the variability observed in homo- and heteroallelic *Ae wx* frequencies, may be the result of analyzing the mutants in nonisogenic backgrounds. Only when the genetic backgrounds are identical, or nearly so, can the presumed *Ae wx* types be attributed more precisely to reversion or suppressor mutations and recombination. On the other hand, lack of complete additivity may routinely characterize intragenic mapping along with nonreciprocal exchange and negative interference, as it does for example in the ascomycetes

(CASE and GILES 1958; LEUPOLD 1958; PRITCHARD 1955; SIDDIQI 1962), where, unlike random gamete analysis, gene conversion can be studied. Recently in fact, gene conversion was demonstrated in a recombinational study of the rosy cistron in Drosophila by utilizing compound-third chromosomes which constitute by analogy to the fungal systems half-tetrads (CHOVNICK *et al.* 1970).

BIANCHI and TOMASSINI (1965) found different frequencies of *wx* reversions in pollen from side branches of maize tassels. Although one would expect this to be reflected in differential recombination frequencies if the same relative spikelet were not routinely sampled, this likelihood was avoided in the present study by sampling only the main spike. Later, BIANCHI (1968) concluded that the occurrence of crossing over in the *wx* region is both heterogeneously distributed in different plants of the same genotype and in different anthers of the same tassel.

Environmental conditions (NELSON 1968; YU and PETERSON 1970), B chromosomes (MELNYCZENKO 1970) and presence of the *Ac-Ds* system of gene controlling elements (NELSON 1968; MCCLINTOCK 1967) are also likely to alter intragenic recombinational values in maize. However, since all samples analyzed in the present study were grown under the same greenhouse conditions, except for day to day fluctuations, environmental conditions were not extremely different during pollen development. Likewise, contamination can be ruled out as a source of increased frequencies of *Ae wx* in these analyses because no pollen of this type was grown in the greenhouse during the study. In any case, rinsing of the tassels prior to storing them in ethanol would have removed the foreign pollen.

In addition, there is no reason to believe that either B chromosomes or *Ac-Ds* were introduced into the lines investigated. In fact, genetic tests for the latter system were conducted after its presence was suspected to be the basis for different recombinational values in a reciprocal cross in preliminary pollen analyses (MOORE 1969), but these tests failed to establish the system's presence. That is, the typical distinct *Ac-Ds* phenotype was never observed in crosses designed to detect the regulatory elements.

In order to properly orient the *ae* region defined in this study on the linkage map of chromosome five, the incorporation of outside genetic markers into *ae wx* homo- and heteroallelic stocks should be completed. At the same time some of the interallelic recombinational estimates derived from these pollen analyses should be substantiated by conventional recombinational procedures, particularly since presumed *Ae wx* pollen types cannot be verified in these analyses as recombinant kernel types should be.

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