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Mutation Research 2 (1965) 339–344

MUTATIONS INDUCED BY ETHYL METHANESULFONATE IN MAIZE

E. AMANO AND H. H. SMITH
Biology Department, Brookhaven National Laboratory, Upton, N.Y. (U.S.A.)
(Received February 15th, 1965)

SUMMARY

"Single-locus mutations" were induced frequently and almost exclusively by treating seeds or young seedlings of *I Sh Wx* stocks of *Zea mays* with aqueous solutions of ethyl methanesulfonate, while X-irradiation of pollen produced a high proportion of "multiple-locus mutations".

INTRODUCTION

One significant outcome of recent mutation studies in higher plants is the finding of a very high mutagenic activity of the alkylating agents ethyl methanesulfonate (EMS) and diethyl sulfate. In a review, NILAN[20] gave a list of frequencies of chlorophyll mutations induced in barley by several mutagens; and, in terms of mutations per 100 M_1 spikes, EMS and diethyl sulfate showed about a three-fold higher frequency than ionizing radiations. The high mutagenicity of these compounds has now been reported in a number of organisms including Drosophila[7], Neurospora[25], yeast[10], bacteria[15,28] and T2 phage[14], as well as in barley[5,8,11]. According to recent studies[1,13] the ethyl group of these two alkylating agents is considered to be responsible for alkylation of DNA bases, and this is followed by biochemical processes which lead

Abbreviation: EMS, ethyl methanesulfonate.

to the ultimate mutation. Some of the chemical reactions involved have been studied with regard to the DNA bases attacked[1,13] and the "strandedness" of the DNA[24].

The high mutation frequency accompanied, under certain conditions, by a low incidence of chromosome aberrations in barley[9] and also in chromosomes of *Vicia faba*[17] has stimulated further studies of induced mutations in the strict sense *vs.* chromosome aberrations in higher plants. Using three closely linked markers on chromosome 3 in maize, NEUFFER AND FICSOR[19] succeeded in obtaining with EMS treatment of young tassels a single kernel, out of 12 131, which showed a mutation only for the middle one of the three markers.

The work reported here involves the use of endosperm marker genes on the short arm of chromosome 9 in *Zea mays* (L.)[16] as a system for testing the mutagenicity of EMS and comparing its effects with those of ionizing radiations in producing chromosome breaks and gene mutations.

MATERIAL AND METHODS

The relative positions of the marker genes used[4,22], are shown in Fig. 1. Although these genes are located some distance apart, they can be used to obtain information on the frequency of induced loss of a large chromosomal segment *vs.* a small intercalary deletion or possible gene mutation[6,12]. Stocks of multiple dominant

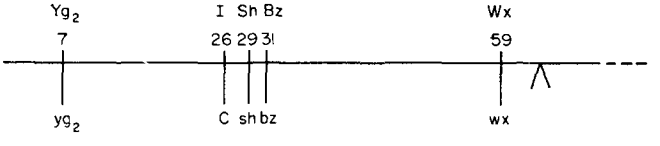

Fig. 1. Diagrammatic representation of the short arm of chromosome 9 showing relative positions of genes used.

homozygotes *(I Sh Wx)* were treated and crossed, as male or female parent, with a corresponding multiple recessive stock *(C sh wx)*. If the dominant genes are lost (deletion) or undergo a change in function (mutation) the recessive character may appear in the F_1 kernel. In this paper any loss in phenotypic expression will be termed a "mutation" in the broader sense.

In addition to changes in the three marker genes noted above, mutations to the gene *bz* were looked for in *C* phenotypes[21], but none were observed. Experiments were also conducted with Yg_2/yg_2 heterozygotes. This material was useful in marking mutant yellow green areas in somatic tissue of treated plants in order to show the size of these areas and as an early indication of the effectiveness of different treatments. The chlorophyll deficient mutant sectors produced ranged in color from slightly yellowish green to white, and their genetic analysis is in progress.

In order to obtain large mutant areas, and to facilitate in establishing sufficient seed stocks of the induced mutants for further study, EMS and radiation treatments were applied mostly to seed embryos or very young seedlings.

EMS treatments

Since EMS becomes hydrolyzed in water over a period of time, and since its chromosome-breaking ability is influenced by impurities in the aqueous solvent, the following precautions were used in preparing EMS solutions. All treatments were begun within an hour after preparation of the solutions. The water used in all experimental procedures was distilled and deionized in order to provide conditions which minimize the production of chromosomal aberrations[17].

Three methods of treatment with EMS were used. (1) *Seed soaking.* The surface of the seeds was first disinfected with a mixture of equal portions of 95% ethanol and 3% hydrogen peroxide. The seeds were then soaked in deionized water at 27° and bubbled continuously with oxygen for 24 h. They were then soaked in 0.05 M, 0.025 M or 0.01 M aqueous solution of EMS for either 5 h at 27° or for 2 to 5 days at 3°. In 1963 the EMS solutions were made up in unbuffered deionized water. In 1964 the solutions were adjusted to a pH of 7.6 with phosphate buffer, and also, Cu^{2+} ($1 \cdot 10^{-3}$ mM) was tested with and without EMS. The rationale for the cold treatments, *i.e.*, for prolonged applications at a cold temperature, was to ensure thorough penetration without chemical disintegration of the mutagen. This was followed by post-incubation in water at different temperatures; however the data are not extensive enough at present to determine if differences in post-treatment temperature influence the results. In this preliminary report the results of all seed soaking treatments are combined, since no conclusive evidence of significant differences attributable to different methods of soaking is yet available.

(2) *Cut root method.* Seeds were prepared in the same manner as for the seed soaking treatment, then placed on slanted moist filter paper to ensure growth of straight roots. When the young roots reached 2 to 5 cm in length, the tips were cut off about 0.5 cm from the end. These young seedlings were then put in glass vials so that the root was immersed in a solution of 0.01 M EMS, while the seed and shoot remained in air for normal respiration. This treatment was continued for 24 h at about 24° and under ordinary room illumination. The purpose of the cut root method was to ensure rapid uptake of the mutagenic solution, and subsequent transport through vascular tissue to the apical stem meristem of the young plant.

(3) *Injection.* Injections of 5 to 10 ml of 0.005 to 0.05 M EMS solutions were made into the lower part of the tassel of young plants with a hypodermic needle and syringe. Test injections with dyes indicated an even distribution of the solution among florets. A few mutants were obtained with EMS by this method; however, treatment of the seed or very young seedlings was found to be more convenient and effective for the purposes of these experiments.

Radiation treatments

Dry seeds were irradiated with reactor-generated fast neutrons; and in separate experiments, pollen from untreated plants was exposed to X-rays. Fast neutron irradiations were carried out in the thermal column of the Brookhaven Graphite Reactor by using a U-235 converter plate. Dosages of 1000, 2000 and 2500 rad were delivered to dry seeds at a rate of 138.5 rad/min. The X-ray treatments were made with a G.E. Maxitron apparatus operated at 250 kVp, 30 mA, 1.0 mm Al filter, and 30 cm target distance. Mature dry pollen was exposed to a total X-ray dose of 1200 R in 1963 and to 1000, 2000 and 3000 R in 1964 at a dose rate of 1272 R/min.

All chemically treated seeds or seedlings were rinsed in water after treatment. Both these and the neutron irradiated seeds were sown in steam-sterilized soil, and grown in the greenhouse until well established before transplanting to the field. At maturity these plants were used as either male or female parents in crosses with a homozygous recessive tester stock. The F_1 kernels were checked for endosperm mutations after harvest and drying.

RESULTS AND DISCUSSION

The results of treatments with EMS and with radiation, as observed in F_1 kernels, are shown in Table I. The data are grouped according to whether the treatment was applied early, *i.e.*, to seeds or young seedlings; or late, *i.e.*, applied to the tassel or pollen. If applications were made at an early stage of embryo or plant development, most mutations appeared in large sectors. This was shown by: (1) the appearance of large chlorophyll deficient sectors in treated plants, particularly in Yg_2/yg_2 heterozygotes; (2) in female reproductive tissue by the appearance of areas of mutant kernels on ears of treated plants pollinated with a recessive tester; and (3) in male reproductive tissue, by large chlorophyll deficient sectors in the tassel of treated plants as well as by a high frequency of mutant endosperm kernels of the same type on ears of recessive tester plants fertilized with pollen from the tassel of treated plants. Therefore, the ear or tassel as a whole was taken as the unit for scoring frequency of mutations produced; each cluster of the same mutation was equated to a single mutation. On the other hand, when treatments were made at later stages, by injection of EMS into young tassels or by X-irradiation of mature pollen, each single mutated kernel among the total number of kernels scored was considered to be an independently induced mutation.

The results shown in detail in Table I are summarized in Table II. Early EMS treatments, *i.e.*, soaking and cut root methods pooled, gave 91 single locus mutations out of 1937. This is a mutation rate of $470 \cdot 10^{-4}$. A few cases of exceptionally high incidence of single locus mutations from early treatments were observed. In one, 4 out of 16 ears, and in another, 8 out of 27 ears, had a mutation in one of the three loci scored. The high incidence of single locus mutations from early EMS treatments and from exposure to fast neutrons may be due in part to consequences of severe screening of large chromosomal deletions during development of the plant tissue (diploidal elimination) or in reproductive stages (haploidal elimination). However, only one multiple locus mutation, compared to 26 single locus ones out of 21665 kernels was obtained from tassel injections with EMS. X-irradiation of pollen produced almost the same frequency of single ($31 \cdot 10^{-4}$) and multiple locus mutations ($29 \cdot 10^{-4}$). There was no clear evidence that either buffering the EMS solution or presence of Cu^{2+} affected mutation rate.

The mutations listed in Table I were induced in 1963 and 1964, and a representative sample (1 to 5) of 1963 mutant kernels, with the exception of those induced by X-ray treatment of pollen, were sown in 1964 to confirm the mutations by testing their transmission (Table III). This was particularly important with *C* and *sh* mutations which may not always be clearly identifiable. Two doses of *C* in an endosperm may give some coloring in the presence of *I* (ref. 2), and the expression of the *sh* phenotype may be influenced by nutritional conditions during development. Of the

24 colored (non-*I*) mutations from treated material, all germinated and 16 or 17 were confirmed as *c*-type mutations. Of the 28 *sh* mutations tested, 24 germinated and 19 were confirmed as transmissible. One of those not transmitted was from a tassel injection treatment and may have been due to non-correspondence between two generative nuclei that were produced by division occurring prior to the mutation event. Of the 31 *wx* mutations tested, all germinated, and all but two were transmitted to the next

TABLE I

WHOLE KERNEL MUTATIONS INDUCED BY EMS AND RADIATION IN MAIZE

Mutagen	*Year*	*Method or material*		*Total number of:*	*Number of mutations*			
					Single			*Multiple*
					C	*sh*	*wx*	
Seed or seedling treatment				*Ears and/ or tassels*				
Fast neutrons	1963	dry seed	2000 rad	228	3	1	1	0
	1964	dry seed	1000 rad	353	0	0	0	0
			2000 rad	253	3	0	3	0
			2500 rad	253	0	3	0	0
	1963–4			1087	6	4	4	0
EMS	1963	soaking	d. H_2O	1158	18	20	24	1*
		cut root	d. H_2O	149	3	5	2	0
		soaking	control	502	1**	0	0	0
		cut root	control	58	0	0	0	0
	1964	soaking	Cu^{2+}	166	0	1	1	0
			buffer	362	0	3	6	0
		cut root	d. H_2O	102	2	2	4	0
		control	Cu^{2+}	250	0	0	0	0
			buffer	258	0	0	0	0
			d. H_2O	88	0	0	0	0
	1963–4	treated		1937	23	31	37	1
		control		1156	1	0	0	0
Tassel or pollen treatment				*Kernels*				
X-ray	1963	pollen	1200 R	1004	1	1	1	6
	1964	pollen	1000 R	826	2	0	1	0
			2000 R	583	0	0	0	0
			3000 R	1748	3	3	1	6
	1963–4	treated		4161	6	4	3	12
	1963	pollen	control	181	0	0	0	0
	1964	pollen	control	487	0	0	0	0
	1963–4	control		668	0	0	0	0
EMS	1963	tassel injection	d. H_2O	4994	0	2	3***	0
	1964	tassel injection	Cu^{2+}	3751	0	3	4	1
			buffer	2443	3	0	1	0
			d. H_2O	10477	3	4	3	0
	1963–4	treated		21665	6	9	11	1
	1963	control	d. H_2O	471	0	0	0	1****
	1964	control	Cu^{2+}	4018	0	0	0	1
			buffer	1893	0	0	0	0
			d. H_2O	4382	0	0	0	0
	1963–4	control		10764	0	0	0	2

Abbreviation: d. H_2O, deionized water.

* *sh wx*, but only *wx* appeared in next generation.

** The plant grown from this was weak and produced no seed.

*** Two of these kernels came from the same treated tassel.

**** *C sh wx*, but only *wx* appeared in next generation.

TABLE II

SUMMARY OF INDUCED MUTATION RATES

Application time	*Mutagen*	*n*	*Mutation rate* $\times 10^{-4}$	
			Single	*Multiple*
Early	Fast neutrons	1087	128.80	0.00
	EMS	1937	469.80	5.16
	Control	1156	8.65	0.00
Late	X-ray	4161	31.24	28.84
	EMS	21665	12.00	0.46
	Control	11432	0.00	1.75

TABLE III

PROGENY TEST CONFIRMATION OF INDUCED MUTATIONS

Treatment	*Number of mutations observed*	*Number of tested mutations germinated*	*Number of mutations confirmed*
wx mutations			
Fast neutrons	1	1	0
Early EMS application	27	27	27
EMS injection	3	3	2
sh mutations			
Fast neutrons	1	1	1
Early EMS application	25	21	17
EMS injection	2	2	1
c mutations			
Fast neutrons	3	3	2
Early EMS application	21	21	14 (15)
EMS injection	0	—	—

generation. One of these was induced by fast neutrons and was accompanied by loss of all other markers on half the kernel. The other was from late EMS injection and may have been due to non-correspondence of mutation in the two generative nuclei as noted above.

Pollen fertility in the F_1 of the 29 transmissible *wx* mutations induced by EMS was checked under low magnification after staining with I_2–KI. More than 90% fertility was found in 22 of these *wx* mutants. Of the 18 *sh* mutations produced by EMS treatment 17 were tested for pollen fertility and 16 were found to be more than 90% fertile. Of the 14 definitely confirmed *c* mutations produced by EMS, all were tested and 13 showed more than 90% pollen fertility. In the *IWx* → *cWx* mutant, which was of low fertility in the F_1 with the *C sh wx* tester, the cause of pollen abortion appeared to be independent of chromosome 9 since the ratio of *Wx* (black) to *wx* (brown) grains was approximately normal.

While checking the stained pollen of *wx* mutants, it was noticed that in 14 of them the pollen had a different shade of brown color. About half the grains in the F_1's with the *wx* tester stock were stained darker brown than the other half; but were definitely brown compared to black-staining *Wx* grains in the same preparation. In the endosperm no difference in *wx* expression among *wx* mutants has so far been observed.

A question of primary interest is whether the mutations induced by EMS are gross changes (deletions) or "point" mutations. A fine structure analysis can be applied

to the *wx* mutants since the *wx* character can be scored in large numbers in the pollen. Intra-cistron recombinants are detectable[18], with iodine staining, as black *Wx* grains occurring at frequencies above backmutation levels. Most of the *wx* mutants which have been crossed to *wx* tester stock have shown a few black-staining *Wx* pollen grains in the F_1. Efforts are now being made to map all the new *wx* mutations.

All confirmed "non-*I* mutants", which have been crossed with the *C* tester stock and selfed, segregated for colorless kernels. This result was interpreted as an indication that the mutations were not $I \rightarrow C$, but rather, were either $I \rightarrow c$ or a deletion of *I*. While the latter is a more likely interpretation, two arguments for expecting relatively frequent $I \rightarrow c$ gene mutations may be mentioned. If the locus is bifunctional[3] and *c* represents the absence of complete gene products produced by *I* and/or *C*, then incomplete gene products might be caused by a number of different alterations in the locus. As a consequence, *c* mutations would be the more frequent. Secondly, if the locus were compound, which appears unlikely[3], and if the treated material was *Ic* rather than *IC*, then the most frequent mutations might be expected to be to colorless *ic*.

The interpretations that true gene mutations, free of major change in chromosome structure, may be produced in *Zea mays* by EMS is encouraged by: (1) induction almost exclusively of single locus mutations, (2) the high fertility of most mutants and normal segregation of chromosome 9 markers in some, (3) alternative explanations to deletion for the $I \rightarrow c$ mutations, and (4) preliminary evidence of recombination among induced *wx* mutants.

With early applications of the mutagen, *i.e.*, treatment of seeds or seedlings, all mutations were expressed throughout the whole kernel; and, in the case of ears formed on treated plants, a ratio of approx. 1 entirely mutated kernel: 1 normal kernel was usually found in the area of the ear containing mutations. However, with late application of the mutagen, *i.e.*, treatment of the tassel or pollen, several kernels showed only a sector of mutant endosperm. These partial mutants or deletions are not included in the data presented in Table I. For example, in the 1964 experiments, ten fractionally mutated kernels (with more than 1/8 of the seed surface mutated) were found. Of these, nine (from both EMS and X-ray treatments) showed a loss of *I*, *Sh*, *Bz* and *Wx* and only one (from buffered EMS treatment) showed a single marker *(wx)* mutation. Other evidence of chromosome breakage was observed as mosaic-patterned kernels resulting from a breakage–fusion–bridge cycle. Nine were found in the material from tassel injections of EMS and in all of these the initiating points of the breakage–fusion–bridge cycle were distal to *I*, as was also observed to occur occasionally in controls. X-irradiation of pollen induced breakage–fusion–bridge cycles in which the distribution of initiating points was proportional to the map distances between the markers used. Data on these chromosome breaks are not included with the whole kernel mutation data in Table I since the main emphasis in this paper is on the EMS-induced mutants.

ACKNOWLEDGEMENT

Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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