EFFECTS OF X RAYS AND ETHYL METHANESULFONATE ON THE CHLOROPHYLL *B* LOCUS IN THE SOMA AND ON THE THIAMINE LOCI IN THE GERMLINE OF ARABIDOPSIS^{1,2}

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Received May 2, 1968

IN higher plants the mutagen is often targeted to premeiotic or postmeiotic cells of the germline and the mutations are scored in the following sexual generations. Alternatively, heterozygous somatic tissues are studied for the expression of the recessive phenotype after mutagenic treatments. This latter technique yields results rapidly since the consequence of the treatment can be readily assessed in the same generation; furthermore, it is quite economical since in each individual a large number of initial cells may contribute to sectors as a result of genetic alterations. The locus specificity of the procedure is considered as an additional advantage.

It should be borne in mind that the somatic mutation technique classifies locus specificity on the basis of phenotype alone and as will be shown below, visual classification is often loaded with great error. The estimation of mutagenic effectiveness at specific loci in the somatic cells may give results different from those in the germline because unspecific dominant lethals and physiological effects complicate the identification. Point mutation is lumped with deletion, somatic recombination, nondisjunction, translocation, the consequences of breakage-fusion-bridge cycles (cf. JONES 1937; McCLINTOCK 1951) and a number of other phenomena. The studies of somatic mutation reported in the literature do not always discriminate experimentally among these possibilities.

Though the investigations reported below had been designed to study somatic recombination and genetic control of thiamine synthesis, by-product information has been obtained on the comparative efficiency of X rays and ethyl methanesulfonate to produce genetic alterations at specific loci in soma and germline.

MATERIALS AND METHODS

X rays (150 kv, 9 mA) and ethyl methanesulfonate (EMS; Eastman, Rochester, N.Y.) were used as mutagens on *Arabidopsis thaliana* (L.) Heynh.

Somatic sectors were induced with 13 kR delivered to seed presoaked for 24 hrs or by submerging dry seed for 15 hrs in various concentrations of EMS as given in Table 1. Germline mutations were induced in dry seed with 50 kR or with 0.2% EMS treatment for 15 hrs. Previous observations showed that the two different X ray treatments are about equally effective on

¹ Contribution from the Missouri Agricultural Experiment Station Journal Series No. 5401. Approved by Director.

² This research was supported by the U.S. Atomic Energy Commission Contract AT(11-1) 1609 and National Science Foundation Grants GB3999 and GB6577.

FIGURE 1.—Typical sectorial plant of *Arabidopsis* observed under the experimental conditions. Approximately 1/16 of the rosette was "mutant," indicating that the vegetative apex was represented by 16 "functional" cells at the time of the mutagenic treatment.

Arabidopsis. The lowest concentration of EMS (0.25%) used in the experiments where somatic effects were scored was close to that used for the study of the germline effects (0.20%).

The mutagen treated seeds were suspended in 0.12% agar, 25 per ml. The free-flowing suspension was applied as drops to the surface of the soil or distributed as a very fine continuous flow from a separatory funnel to give a density of 30–50 seeds per 13 cm pot. All cultures were grown in the greenhouse.

Somatic effect of the mutagens was scored in F_2 populations where 50% of the cross $gi^1ch^1 \times gi^2pa\ er$ was heterozygous for the *ch* locus. Genetic alterations in the soma were assessed by the number of plants displaying *ch* sectors. In homo- or hemizygous condition the *ch* tissues are free of chlorophyll-*b* and can be easily recognized by the yellow green color. The identity of a sector can be ascertained by thin-layer or paper chromatography (Figures 1 and 2). The sectors were

TABLE 1

Frequency of induced somatic sectorials among ch heterozygotes

Dose kR	Survivors	X rays			Dose percent	Survivors	Ethyl methanesulfonate		
		Phenotype of sectorials percent		Proven <i>ch/ch</i> or <i>ch/0</i> sectorials percent			Phenotype of sectorials percent		Proven <i>ch/ch</i> or <i>ch/0</i> sectorials percent
		<i>non-ch</i>	<i>ch</i>				<i>non-ch</i>	<i>ch</i>	
13	1317	0.25	1.22	0.68	0.50	1056	15.05	4.35	1.42
13	1434	0.35	0.63	0.49	0.35	1316	3.88	1.75	0.76
13	1122	0.36	0.89	0.89	0.30	1702	1.11	0.65	0.29
13	1795	0.77	1.78	1.78	0.25	1327	2.57	0.90	0.15
Total	5668	0.43	1.22	1.02	Total	5401	4.87	1.70	0.57

carefully cut from the plant, macerated with a glass rod and extracted in two or three drops of acetone in 8×75 mm test tubes. The extract was spotted on freshly made 0.25 mm thin layers of MN 300 cellulose powder on 20×20 cm glass plates, or on fresh Polygram Cel 300 precoated plastic sheets (Brinkman Instr., Westbury, N.Y.). The separation of leaf pigments took about 10–15 min on the glass and only a third of that time on the coated plastic sheets. The chromatogram was developed in the dark in cold glass tanks containing a mixture of petrolether:acetone (10:1.2, v/v). In this system chlorophyll-*b* appears distinctly below chlorophyll-*a* with little tailing. Since chlorophyll-*b* displays bright red fluorescence under short wave length ultraviolet light (Mineralight USV-12, Ultraviolet Products, San Gabriel, Calif.), it can be distinguished with certainty from the purple chlorophyll-*a*.

The frequency of genetic alterations at the *ch* locus in the soma is expressed on a genome basis in order to make it comparable to that of the germline (sectorials/heterozygotes $\times$ 16 cells $\times$ 1 genome). At the time of the mutagen treatment the vegetative meristem was represented by 16 heterozygous cells (Figure 1).

Alleles *gi*¹ and *gi*² delay flowering and give larger rosette leaves which facilitates identification of the *ch* sectors, even in cultures grown under continuous illumination. Genes *pa* and *er* are not pertinent to the following discussions.

Germline effects of the mutagens were scored by the frequency of thiamine auxotrophs obtained at four loci. The treated material was homozygous recessive for certain markers to identify possible contaminations, but all thiamine loci were represented by the wild-type alleles only. The auxotrophs were isolated in soil cultures where the characteristic symptoms of thiamine deficiency can be easily recognized (Figure 3). The exact nutritional requirements were ascertained later in test tubes on chemically defined aseptic media. Mutation frequency in the germline is expressed as an average per four loci (mutants/survivors $\times$ 2 cells $\times$ 2 genomes $\times$ 4 loci). Under the conditions of the experiments the germline was represented by two diploid cells as detected by the M_2 segregation ratios. Details of the method of estimation of mutation rate will appear elsewhere (Li and RÉDEI 1968).

RESULTS

When germline mutations or somatic sectors were scored after treatment of presoaked seeds with 13 kR or dry seeds with 50 kR, approximately the same genetic effects were observed as judged by the number of chlorophyll mutations at a large number of loci or by the number of leaf sectors in heterozygotes for a few visible markers. Thus comparison of the EMS effect to that of X rays in both series of experiments is not arbitrary even if the absolute doses of X rays were different.

FIGURE 2.—Separation of leaf pigments by paper chromatography. Left from wild type, right from chlorophyll-*b* free tissues. Lines from bottom: start (chlorophyllides), chlorophyll-*b*, chlorophyll-*a*, xanthophylls, carotenes. The paper chromatogram was contact printed on sensitive film and copied on photographic paper.

Chlorophyll-*b* deficient leaf sectors can be easily identified; chimeras for thiamine requirement are not detectable somatically because of the easy diffusion of the vitamin through the tissues (cf. RÉDEI 1967). By using the method of bulk planting the isolation of chlorophyll-*b* mutants is loaded with considerable error. Some of the weak, pale seedlings may be lost before identification becomes possible. A practical comparison can be made, however, if somatic alterations are estimated by the frequency of *ch* sectors in heterozygotes and mutation frequency in the germline is assessed by the number of independent families segregating for thiamine auxotrophy. Within the series of treatments there was not much difference in survival according to the mutagenic treatments used. It was clear that ethyl methanesulfonate in all doses was much more effective than X rays in the production of yellow sectors. In higher concentrations, the chemical induced a considerable number of segments completely or almost completely free of pigment. At lower concentrations the number of "white" sectorials was about the same in the EMS- and X-ray-series. By phenotypic classification, the frequency of

FIGURE 3.—A portion of a 13 cm pot containing the bulked second generation progenies of about 25 mutagen treated seeds. The surface of the soil was sprayed with a minimal amount of a thiamine solution in order to keep alive the thiamine mutants until identification is possible. The tooth-pick marked auxotrophs were then cured and maintained by continuous supply of the vitamin.

apparently chlorophyll-*b* deficient sectorials was from 1/3 to 15 fold higher among the chemically treated plants depending on the dose used (Table 1).

A chromatographic analysis on 55 sectors of *ch* phenotype in the X-rayed series revealed that about 84% were indeed chlorophyll-*b* free, while from the 53 sectorials of the EMS series, though appearing phenotypically *ch*, only 34% proved to be really deficient in chlorophyll-*b*. Only the concentrations of EMS higher

TABLE 2

Average frequency of induced mutation per genome in the germline for thiamine auxotrophy at four loci and induced frequency of chlorophyll-b free sectors per genome in the somatic cells of the embryo

Mutagen	Chlorophyll-b free sectorials		Thiamine auxotrophs	
	Total number	Frequency	Total number	Frequency
X rays	58	6.4×10^{-4}	11	5.4×10^{-5}
EMS	31	3.6×10^{-4}	51	7.8×10^{-5}

than 0.3% induced chlorophyll-*b* free sectors in frequencies comparable to that of X irradiation of 13 kR.

At the low concentration of 0.2% EMS induced an essentially similar or somewhat higher frequency of thiamine mutants compared to X rays. In the somatic experiments only the highest concentration of EMS appeared to be somewhat more effective than X rays to produce true chlorophyll-*b* deficient sectors. The efficiency of EMS and X rays was not very much different within the somatic or the germline experiments (Table 2). On the basis of simple visual classification of the sectors, EMS might have been considered significantly more efficient than X rays (Table 1). The number of somatic alterations, however, is significantly higher than the number of germline mutations induced by both mutagens.

DISCUSSION

The data presented here were obtained from experiments designed for purposes other than the study of mutagenic action. Primarily qualitative information was obtained because of the differences in treatment.

Somatic sectors were induced by 13 kR X rays delivered to 24 hr presoaked seed, whereas dry seeds were treated with 50 kR to produce alterations in the germline. The two kinds of treatments do not differ much if either somatic effects or the germline effects are used for the comparison. On a genome basis, the somatic effects of X rays are an order of magnitude larger at the *ch* locus than the average effect at the thiamine loci in the germline.

Differences in dosage effects can be readily estimated from the data obtained from EMS treatments. The frequency of *ch* sectors in the four concentrations of EMS indicates a sharp increase with dose.

According to these studies only a fraction of the total somatic genetic effects can be attributed to point mutations as defined by STADLER (1944). Consequently, mutagenic efficiency of unknown agents can not be determined and compared on the basis of somatic tests.

The experiments clearly indicate that somatic tests can meet the locus specificity requirement only if the identity of the sector (e.g. *ch/ch* or *ch/0*) can be determined with greater certainty than permitted by simple phenotypic classification. In the present experiments the effectiveness of EMS in producing locus specific sectors would have been estimated by a threefold error if chromatographic analy-

sis would not have been used (cf. Table 1). The error might have been reduced by planting parallel with the *ch*/+ population a control of equal number of +/+ plants and counting only the difference in the number of sectors as done by FAVRET (1960). The inconvenience of such a procedure is obvious. Since EMS induced 5–25 times as many unspecific sectors as the number of chlorophyll-*b* free ones (Table 1), a subtraction procedure may have indicated locus specificity of the mutagen if there were fluctuations in the control.

The first exploratory studies on somatic alterations were made in 1963 in collaboration with Dr. Y. HIRONO and his contribution is greatly appreciated. Thanks are due to Dr. G. RÖBBELLEN for his assistance in some experiments carried out in 1966.

SUMMARY

The frequency of observable somatic alterations and germline mutations has been studied at specific loci of *Arabidopsis* after treatments with X rays and ethyl methanesulfonate (EMS). Identity of the somatic sectors was determined chromatographically. Mutants of the germline were identified by nutritional requirement (thiamine auxotrophy) and allelism tests. Under the experimental conditions X rays induced more somatic alterations compared to the frequency of apparent point mutations. EMS and X rays induced approximately equal numbers of genetic changes in germline and soma. By phenotypic classification alone EMS appeared more effective than X rays in the induction of sectoring concerned with a specific locus. The chromatographic analysis revealed, however, that in the EMS series most of the visually classified *ch* sectors contained substantial amounts of chlorophyll-*b*, while in the X ray series the majority of the *ch* appearing sectors contained only chlorophyll-*a*. The experiments showed that phenotypic classification of body sectors is unreliable for testing genetic alterations at specific loci, but chemical tests as used in *Arabidopsis* give meaningful estimates.

LITERATURE CITED

- FAVRET, E. A., 1960 Somatic mutations of four genes for albinism in barley induced by X rays and ethyl methanesulphonate. *Hereditas* **46**: 622–633.
- JONES, D. F., 1937 Somatic segregation and its relation to atypical growth. *Genetics* **22**: 484–522.
- LI, S. L., and G. P. RÉDEL, 1969 Estimation of mutation rates in autogamous diploids. *Radiation Botany* **9**: 125.
- McCLINTOCK, B., 1951 Chromosome organization and genetic expression. Cold Spring Harbor Symp. Quant. Biol. **16**: 13–47.
- RÉDEL, G. P., 1967 Genetic estimate of cellular autarky. *Experientia* **23**: 584.
- STADLER, L. J., 1928 Genetic effects of X rays in maize. *Proc. Natl. Acad. Sci. U.S.* **14**: 69–75.
- 1944 The effects of X rays upon dominant mutation in maize. *Proc. Natl. Acad. Sci. U.S.* **30**: 123–128.