Combining cytogenetics and mutagenesis to recover unusual mutants at specific loci in barley (Hordeum vulgare L.)
by Daniel Reid Biggerstaff

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
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Abstract:
Seed of translocation heterozygotes in barley (Hordeum vulgare L.) from crosses between male steriles and selected translocation homozygotes was treated with the chemical mutagen diethylsulphate. Translocations were selected to represent breakpoints in the 14 barley chromosome arms, with the breakpoints as far from the centromere as possible. The male sterile genes selected were located near the centromere of one chromosome involved in the translocation. Diethylsulphate was the mutagen of choice because it produces a high number of point mutations and relatively few chromosomal aberrations, is quite economical, and is easy to use.

M2 head rows were selected on the basis of aberrant ratios with respect to fertility. The expected ratio is 1 fertile:2 semisterile:1 male sterile; the aberrant ratio selected was 0:2:1, representing possible lethal translocation homozygotes. The frequency of recovered mutants in the interstitial segment was approximately 6%. Twenty-three lethal translocation homozygote mutants have been recovered. At least one breakpoint in each of the 14 barley chromosome arms, and all but six of the possible chromosome translocation combinations are represented.

Uses and advantages of lethal translocation homozygotes include: increasing the precision of linkage studies, maintaining recessive genes in heterozygous stocks, accomplishing gene transfers, and allelism testing with greater ease and confidence. Success in recovering mutations in specified regions of the barley chromosome, in coupling or repulsion, was much easier than anticipated. Methods such as this may offer one way of incorporating more of the available cytogenetic and genetic information into the art and science of plant breeding.

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COMBINING CYTOGENETICS AND MUTAGENESIS TO RECOVER UNUSUAL MUTANTS AT SPECIFIC LOCI IN BARLEY (*HORDEUM VULGARE* L.)

by

DANIEL REID BIGGERSTAFF

A thesis submitted in partial fulfillment
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of

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in

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ABSTRACT

Seed of F_1 translocation heterozygotes in barley (Hordeum vulgare L.) from crosses between male steriles and selected translocation homozygotes was treated with the chemical mutagen diethylsulphate. Translocations were selected to represent breakpoints in the 14 barley chromosome arms, with the breakpoints as far from the centromere as possible. The male sterile genes selected were located near the centromere of one chromosome involved in the translocation. Diethylsulphate was the mutagen of choice because it produces a high number of point mutations and relatively few chromosomal aberrations, is quite economical, and is easy to use.

M_2 head rows were selected on the basis of aberrant ratios with respect to fertility. The expected ratio is 1 fertile:2 semisterile:1 male sterile; the aberrant ratio selected was 0:2:1, representing possible lethal translocation homozygotes. The frequency of recovered mutants in the interstitial segment was approximately 6%. Twenty-three lethal translocation homozygote mutants have been recovered. At least one breakpoint in each of the 14 barley chromosome arms, and all but six of the possible chromosome translocation combinations are represented.

Uses and advantages of lethal translocation homozygotes include: increasing the precision of linkage studies, maintaining recessive genes in heterozygous stocks, accomplishing gene transfers, and allelism testing with greater ease and confidence. Success in recovering mutations in specified regions of the barley chromosome, in coupling or repulsion, was much easier than anticipated. Methods such as this may offer one way of incorporating more of the available cytogenetic and genetic information into the art and science of plant breeding.

INTRODUCTION

Barley (Hordeum vulgare L.) is one of the first crops domesticated by man and is grown throughout the world as a major cereal crop. It is also used extensively for genetic studies, in mutagen experiments, and as a laboratory organism for physiologists, biochemists, and others. Because barley is an important commercial crop and is of great interest to scientists from many disciplines, any novel use of barley in a genetic study has far reaching implications.

The purpose of this study was to induce and recover mutant genes at specific loci. A translocation heterozygote having a lethal gene on the interstitial segment of the translocated chromosome, and a recessive male sterile gene near the centromere of the non-translocated chromosome, would provide barley breeders with a useful genetic tool. This lethal translocation homozygote would facilitate gene transfers, increase the speed and precision of linkage studies, and give breeders another way to maintain recessive genes in a known heterozygous condition. Success in this attempt would also serve to demonstrate the potential of combining mutagenesis and cytogenetics to obtain mutants at specific loci.

LITERATURE REVIEW

Genetic male sterility in barley was first reported by Suneson (1940). Since then 377 different male sterile mutants in spring barley have been reported and 33 have been determined to be non-allelic (Hockett, 1972; Hockett, et al., 1968; Hockett and Reid, 1981). Numerous workers have reported chromosomal location of these mutants (Eslick, 1976; Eslick, et al., 1974; Jarvi and Eslick, 1967; Ramage and Eslick, 1975; Tuleen, 1971). Eslick (1971), Hockett and Eslick (1971), and Roath and Hockett (1971) have presented additional information about origin, inheritance, pollen and anther morphology, and possible uses of genetic male sterile mutants.

Barley has been used extensively in studies of chemical mutagenesis (Constantin, 1975; Nilan, et al., 1963; Nilan, 1964). Probably more information is available on mutation induction in barley than in any other crop (Nilan, 1974). Diethylsulphate has been used successfully in barley mutation work because it induces a high number of point mutations with relatively few chromosomal aberrations (Constantin, 1975; Nilan, et al., 1963). Scholz (1971) presents a review of the utilization of induced mutations in barley.

A chromosomal aberration where terminal segments of nonhomologous chromosomes have exchanged positions is known as a chromosomal interchange, a reciprocal translocation, or simply a translocation.

Burnham (1962) and Ramage (1963) provide excellent discussions of the behavior and utilization of translocations. Zecevic (1975) presents an extensive literature review on barley translocations.

Following anaphase I, adjacent disjunction of the chromosomes involved in a translocation will result in spores that abort because of chromosomal duplications and deficiencies. The aborted spores serve as a phenotypic character, termed semisterility, by which the translocation heterozygote can be recognized (Ramage, 1963). When interstitial crossing over is followed by alternate disjunction, the crossover chromatids are in the spores that abort. In barley, which exhibits an excess of alternate disjunction, recovered crossovers are greatly reduced (Kramer and Blander, 1961).

The potential uses for the mutants being sought in this study were proposed by Eslick (1972). Initial work on this study was reported by Biggerstaff and Eslick (1978).

MATERIALS AND METHODS

Selected translocation homozygotes were crossed to genetic male steriles. Translocations were selected to represent breakpoints in the fourteen barley chromosome arms, with the breakpoints as far from the centromere as possible, as determined from the literature (Table 1). Seed of all the translocations used in this study were provided by Dr. R. T. Ramage, University of Arizona, Tucson, Arizona. Most of the genetic male sterile genes used are located near the centromere of one of the chromosomes involved in the reciprocal translocation (Table 2).

The F_1 progeny of these crosses were grown in a winter nursery at Mesa, Arizona, to obtain maximum seed increase. F_2 seed from each cross was treated with 0.015 M diethylsulfate using procedures developed at Washington State University, Pullman, Washington (Nilan, et al., 1963). This procedure is outlined in the Appendix.

Treated seed (M_1 seed, F_2 generation) were space planted in rows 30 cm apart to obtain populations of plants 5 to 8 cm apart within rows. M_2 seed was harvested from individual spikes taken at random in each population. M_2 head rows were planted as single, space planted rows 3 m in length, 30 cm apart.

At maturity each M_2 head row was examined for the absence of fully fertile plants. The expected ratio is 1 fertile (translocation homozygote):2 semisterile (translocation heterozygote):1 male sterile

Table 1. Description of translocations utilized to establish tester stocks.

Translocation Designation	Cultivar	Breakpoints†		Authority
T1-3e	Bonus	L	L	Persson (1970)
T1-4e	Bonus	L	S	Persson (1970)
T1-5f	Bonus	S	L	Ramage et al. (1961)
T1-6a	Mars	S	Sat	Ramage et al. (1961), Tuleen (1974)
T1-6e	Bonus	L	S?	Persson (1970), Nilan (1964)
T1-6j	Bonus	L	Sat	Persson (1970), Ramage (1971)
T1-7c	Mars	S?	Sat	Nilan (1964, Ramage and Suneson (1961)
T1-7i	Bonus, ert a	L	Sat	Ramage (1971)
T1-7k	Bonus, ert a	L	L	Persson (1970)
T2-3a	Gull	S?	S	Kasha and Burnham (1965)
T2-3c	Bonus	S?	L	Kasha and Burnham (1965)
T2-4a	Mars	-	-	
T2-4d	Bonus	S?	L	Ramage et al. (1961)
T2-5a	Bonus	L?	S?	Ramage et al. (1961)
T2-5e	Bonus	-	-	
T3-4b	Mars	-	S	Nilan (1964)
T3-4d	Bonus	-	-	
T4-5e	Bonus	L	L	Persson (1970), Ramage et al. (1961)
T4-7b	Bonus	S	Sat	Ramage et al. (1961)
T5-6b	Bonus	L	L	Hagbert et al. (1975)
T5-7g	Bonus	L	L	Tuleen (1974)
T6-7c	Bonus	S	S	Ramage et al. (1961), Ramage and Suneson (1961)
T3-7c+	Bonus	S	L	Kasha and Burnham (1965)
3-7d	Bonus	L	S	Kasha and Burnham (1965)

S = short arm; L = long arm; Sat = satellite; ? = probably in that arm

- = breakpoint not determined.

†Interchange chromosome with lower number listed first, higher number listed second.

Table 2. Description of genetic male steriles utilized to establish tester stocks.

Cultivar	CI No.	Symbol	Gene Location	Authority
Betzes	6398	msg1	near centromere of 5	Ramage (1966)
Compana	5438	msg2	near centromere of 2	Ramage (1966)
Carlsberg II	10114	msg5	near centromere of 3	Hockett and Eslick (1971)
Heines Hanna	9532	msg6	near centromere of 6	Eslick et al. (1974)
Compana	5438	msg10	near centromere of 1	Hockett and Eslick (1971), Eslick (1976)
Betzes	6398	msg14	near centromere of 1	Hockett and Eslick (1971), Eslick (1976)
Betzes	6398	msg16	on chromosome 7	Hockett and Eslick (1971)
Compana	5438	msg18	on chromosome 7	Ramage and Eslick (1975)
Intro.(Russia)	14393	msg19	near centromere of 7	Hockett and Eslick (1971)
Glacier/Compana	10861	msg22	on chromosome 1	Hockett (1972), Eslick (1976)
Betzes	6398	msg24	near centromere of 4	Jarvi and Eslick (1967)
Betzes	6398	msg25	near centromere of 4	Eslick (1971)
Betzes	6398	msg32	near centromere of 1	Eslick (1976)
Betzes	6398	msg,,bk	on chromosome 6	Eslick et al. (1974)
Betzes	6398	msg,,z	on chromosome 7	Eslick (1971)

genetic (normal homozygote)(Figure 1). Semisterile plants, usually four, from rows having no fertile plants were harvested individually. M_3 seed from these plants were planted as above. Each plant within a M_3 row was classified for spike fertility to confirm a 0 fertile:2 semisterile:1 male sterile genetic ratio (Figure 2). This sequence was continued through the M_5 generation for selected lines. Lines were selected for acceptable agronomic characteristics in addition to goodness of fit to a 0:2:1 ratio.

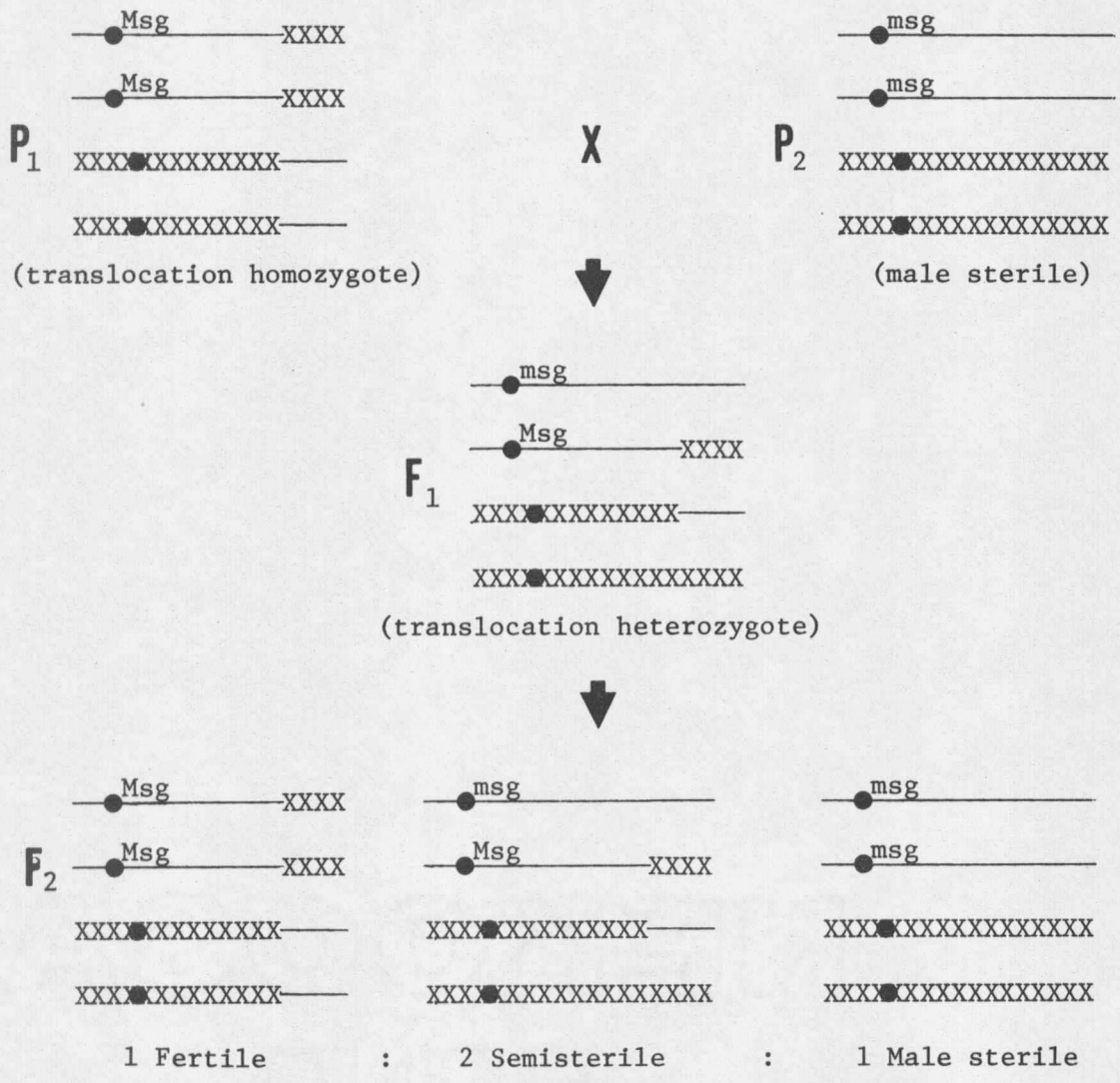

Figure 1. Diagramatic representation of the expected F_2 segregation (chromosomal and genetic) from a cross between a genetic male sterile and a translocation homozygote.

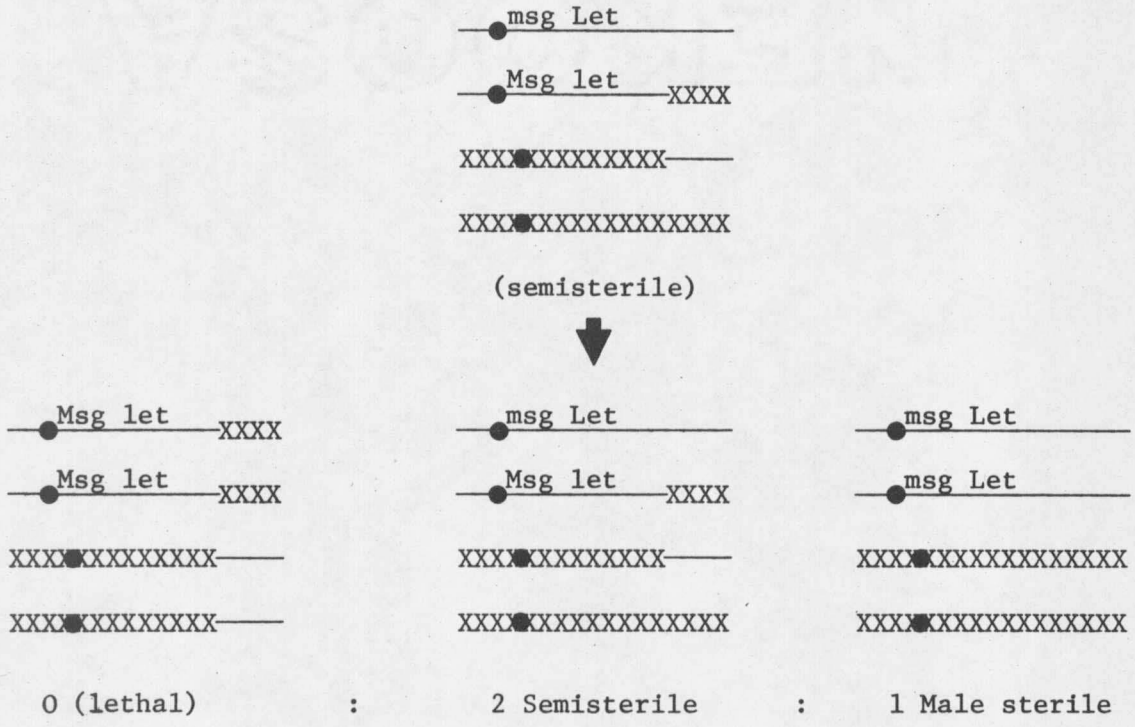

Figure 2. Diagramatic representation of the expected segregation (chromosomal and genetic) in the progeny of a translocation heterozygote having a recessive lethal gene in the interstitial segment, and a recessive male sterile gene proximal to the centromere on the normal chromosome. Msg and let represent hypothetical male sterile genetic and lethal genes, respectively.

RESULTS AND DISCUSSION

The results obtained in this study demonstrate that recessive lethal genes can be induced with diethylsulfate and recovered with the simple classification techniques outlined.

The number of head rows (or lines) selected and continued in each generation is given in Table 3. Out of 4,933 M_2 head rows, 858 appeared to be lacking the fertile (translocation homozygote) class. Most of the M_2 head rows were grown from single spikes on 2-row cultivars, thus the number of surviving plants per row was quite small. Therefore, some of the lines selected did not have fertiles by chance alone, and not due to homozygous recessive lethal genes. Approximately 70 percent of the selected M_3 and M_4 lines exhibited a 0:2:1 ratio with respect to spike fertility. Nearly 80 percent of the M_5 lines closely fit a 0:2:1 ratio. About 6 percent of the original M_2 head rows were found to fit a 0:2:1 ratio in the M_5 generation.

Use of a recessive marker gene in coupling with the translocation breakpoint is a refinement of the general procedure. The T1-7i and T1-7k translocation homozygotes used in this study were also homozygous for the ert-a dense ear locus. Ert-a has been reported to be very near the centromere of chromosome 1 (Persson, 1970), therefore, the absence of erectoides mutants in M_2 head rows was evidence of the lack of translocation homozygotes (fertiles).

Table 3. Number of head rows and lines selected following mutagenesis.

Male Sterile/ Translocation	M_2 Head Rows	M_2 Head Rows Without Fertiles	Selected M_2 Lines Continued	M_3 Lines with 0:2:1 Ratio*	Selected M_3 Lines Continued	M_4 Lines with 0:2:1 Ratio	Selected M_4 Lines Continued	M_5 Lines with 0:2:1 Ratio
msg10/T1-3e	216	76	18	8	8	5	5	4
msg10/T1-4e	216	12	12	3	3	2	2	2
msg10/T1-5f	216	44	16	10	9	7	7	6
msg10/T1-6a	216	37	15	9	9	7	7	5
msg10/T1-6e	290	28	18	12	9	4	4	4
msg10/T1-6j	216	36	15	13	9	7	7	6
msg10/T1-7c	252	26	15	13	9	9	9	8
msg10/T1-7i	216	36	15	7	7	3	3	3
msg10/T1-7k	252	39	16	16	9	8	8	6
msg2/T2-3a	138	25	12	11	9	9	9	8
msg2/T2-3c	200	26	12	12	9	8	8	6
msg2/T2-4a	216	32	16	14	9	3	3	3
msg2/T2-4d	216	56	18	11	9	7	7	5
msg2/T2-5a	216	45	18	16	9	5	5	5
msg2/T2-5e	216	27	14	13	9	5	5	5
msg5/T3-4b	216	24	12	6	6	4	4	1
msg5/T3-4d	97	20	14	14	9	7	7	6
msg1/T4-5e	216	68	18	14	9	7	7	7
msg19/T4-7b	216	20	12	10	9	9	6	3
msg1/T5-6b	252	50	18	10	9	4	4	2
msg1/T5-7g	216	29	13	9	9	5	5	3
msg6/T6-7c	216	49	18	14	9	7	7	7
msg18/T3-7c+ 3-7d	212	53	24	10	9	6	6	2

*Fertile:Semisterile:Male sterile

Some of the induced lethals are listed in Table 4. The most frequent and readily identified mutants were albinos and non-heading dwarfs. All dwarfs listed were easily distinguished in the tillering stage and none produced seed in our field environments. Further study is suggested to determine the nature of the other lethal mutants. Each mutant family within a translocation designation was assigned a single letter designator to aid in data grouping and seed stock labeling.

Sufficient lines were carried in each generation to allow selection of the best mutant family within each translocation designation. For example, T3-4b, mutant family b, and T5-6b, mutant family c, were replaced in later generations by mutant families e and 1, respectively, because too many fertile plants were being found (Table 4). Eight of the ten fertile plants in T5-7g, mutant family h, were counted in one generation; no fertiles have been found in subsequent generations.

Twenty-three lethal translocation homozygote stocks have been developed, including one with a double translocation, T3-7c+3-7d (Table 4). These mutants represent at least one breakpoint in each of the 14 barley chromosome arms, and include all but six of the 21 possible chromosome translocation combinations (Table 1). This makes them an ideal tester set for linkage studies or from which to select a smaller set.

Since a translocation homozygote in the F_2 from a test cross between a semisterile of a lethal translocation homozygote stock and an

Table 4. Ratios obtained using lethal translocation homozygote tester stocks.

Pedigree and Mutant Family of Current Tester Stock	Identifiable Translocation Homozygote Mutant	Translocation Heterozygote Ratio, F_2, nos.				x^2 for Fit to 2:1 ratio†	Other Male Steriles Used‡
		Mutant	Fertile	Semi-sterile	Male sterile		
msg10/T1-3e/*2 Betzes,d	dwarf	74	3	319	175	.94	
msg14/T1-4e/*2 Betzes,a			0	255	140	.77	msg10,msg22
msg14/T1-5f/*3 Betzes,f	albino	49	4	266	132	.01	msg10,msg32
msg10/T1-6a/*2 Betzes,c			0	137	90	3.78	msg14
msg32/T1-6e/*3 Betzes,a	albino		3	286	162	1.55	msg10,msg14
msg32/T1-6j/*2 Betzes,b			0	272	167	4.13	msg10
msg14/T1-7c/*2 Betzes,k			0	230	165	11.56	msg10
msg32/T1-7i/*2 Betzes,d			1	292	146	.00	msg10
msg10/T1-7k/*2 Betzes,c	dwarf	78	0	327	156	.24	
msg5/T2-3a/*Betzes,b	albino	75	0	164	99	2.08	msg2
msg5/T2-3c/*Betzes,h	albino	81	0	193	105	.47	msg2
msg25/T2-4a/*2 Betzes,c	albino	62	4	168	90	.27	msg2
msg24/T2-4d/*3 Betzes,q			1	249	180	13.10	msg2
msg1/T2-5a/*2 Betzes,v			0	212	125	2.07	msg2
msg1/T2-5e/*3 Betzes,d	albino		1	154	64	1.66	msg2
msg5/T3-4b/*Betzes,b			11	224	77	9.50	msg24
msg5/T3-4b/*Betzes,e			2	59	34	.42	msg24
msg5/T3-4d/*Betzes,o	dwarf	44	0	145	87	1.72	msg24
msg1/T4-5e/*3 Betzes,m	albino		0	196	92	.26	msg25
msg,,z/T4-7b/*3 Betzes,b			6	397	261	11.03	msg16,msg19
msg1/T5-6b/*2 Betzes,c			29	232	120	.09	msg,,bk
msg,,bk/T5-6b/*3 Betzes,1			1	104	62	1.14	msg1
msg1/T5-7b/*2 Betzes,h			10	389	321	40.44	
msg,,bk/T6-7c/*2 Betzes,1	dwarf	29	3	354	173	.06	msg6
msg5/T3-7c+3-7d/*Betzes,c			3	157	104	4.62	msg18

†Semisterile:male sterile

‡Male sterile genes used in crosses to obtain combined observed ratios.

identifiable gene is self rouging (or marked), it can be shown that the average linkage intensity information per classified F_2 individual is greater than it is if normal translocation stocks are used (Hanson, 1952; Hanson and Kramer, 1950; Joachim, 1947). Formulae to facilitate calculating linkage intensity between translocation breakpoints in the lethal translocation homozygote tester stocks and other genes have been derived.[1] These formulae may be incorporated in an existing computer program for calculating maximum likelihood estimates of linkage intensities.

Similarily, if a recessive gene is in coupling or repulsion with the translocation breakpoints of a lethal translation homozygote stock, selection of a semisterile plant would insure the heterozygous condition for that gene.

If the appropriate semisterile from a lethal translocation homozygote stock is crossed with a normal plant, those genes on the normal chromosome region opposite the interstitial segment (Figure 3) will have a reduced opportunity for recombination. Selecting a semisterile plant in the F_1 from such a cross, and selecting a fertile plant from its F_2 progeny, will also effectively transfer those genes.

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[1] Biggerstaff, D. R., unpublished data.

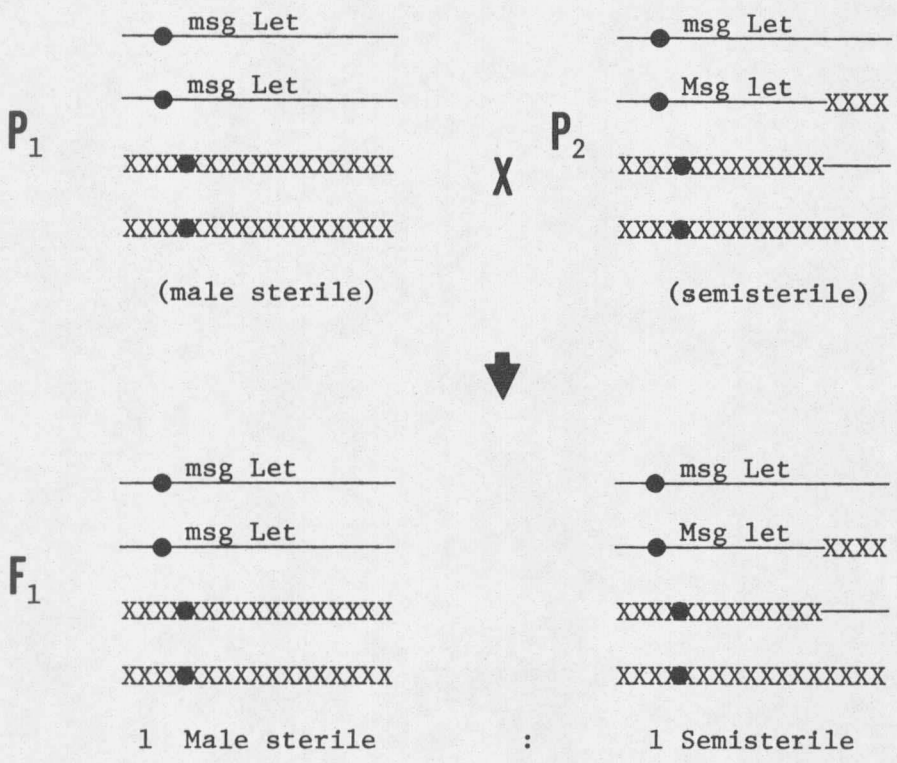

Figure 3. Diagramatic representation of the expected F_1 segregation (chromosomal and genetic) from a cross between a genetic male sterile and a translocation heterozygote. Msg and let represent hypothetical male sterile genetic and lethal genes, respectively. Msg genes are depicted as being allelic.

Rapid allelism tests can be accomplished using the concepts depicted in Figure 3. A cross between a plant, having a known gene homozygous, and the appropriate lethal translocation homozygote stock will produce semisterile progeny with the general genetic and chromosomal configuration of P_2 in Figure 3. If the homozygous recessive gene in P_1 is allelic to the gene on the normal chromosome of P_2, the F_1 will be 50 percent semisterile and 50 percent homozygous for the recessive gene being allele tested (male sterile in the example), and the progeny of semisteriles will segregate 1:1 for the trait and the fertiles will be homozygous for the trait. If the genes are non-allelic, and independent of the interstitial segments of both translocated chromosomes, all F_1 plants will be heterozygous for the trait and their progeny will segregate 9:7 for that character, independent of semisterility or fertility.

The chi-square values for T1-7c, mutant family k, T2-4d, mutant family q, T4-7b, mutant family b, and T5-7g, mutant family h, indicate possible gamete selection. These four lethal translocation homozygote stocks will work satisfactorily for all of the uses mentioned above except calculation of linkage intensities. Since they do not fit a 0:2:1 segregation ratio, linkage intensity values would be distorted.

Available lethal translocation homozygote tester stocks are being backcrossed to the cultivar 'Betzes', in an effort to improve the

agronomic characteristics of these stocks and to achieve some degree of uniformity among them.

The objectives of this study were obtained with much greater ease than anticipated. Perhaps this will serve as a demonstration of the practical utilization of mutagenesis and cytogenetics.

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APPENDIX

TREATMENT PROCEDURES FOR MUTAGEN TREATMENTS ON BARLEY

USED AT WASHINGTON STATE UNIVERSITY

PULLMAN, WASHINGTON

These treatments have been successfully used on Himalaya barley which is a hulless variety. Adjustments should be made for use on a different organism or on a hulled variety of barley.

1. Presoak - The seeds should be flooded with water at 0 C for at least 12 hours to remove any growth inhibitors and to completely hydrate the system. The same effect can be obtained by soaking about 6 hours at 20 C. Allow about 1 ml of distilled water per seed and either use a running water wash or change the water periodically.

2. Pretreatment - This step is designed to get the mutagen into the system without much metabolic activity. The seeds are soaked in the mutagen solution prepared in 0.1 M pH 7 phosphate buffer for 6 hours at 0 C. The concentrations and approximate amount of seedling injury which will be obtained for the three common mutagens are given below:

iPMS - 0.025 M gives about 5% seedling injury and increases gradually up to 0.050 M which gives about 50% seedling injury.

DES - 0.005 M gives about 5% seedling injury and increases gradually up to 0.010 M. Increases to 80% injury at 0.020 M.

EMS - 0.05 M gives about 5 - 10% seedling injury which increases to about 50% seedling injury at 0.25 M.

To prepare the mutagen solution, the mutagen is pipetted into a flask and the buffer is added. This mixture must be shaken very vigorously to get the mutagen into solution. It should be prepared and immediately put on the seeds since the mutagens hydrolyze in the buffer. About 0.5 to 1.0 ml of mutagen solution should be allowed per seed.

3. Treatment - The pretreatment solution should be poured off and a fresh solution added to the seeds. The seeds are then allowed to sit in the mutagen solution for about 2 hours at 20 C. The same solution concentration is used that was used in the pretreatment.

4. Aftertreatment - The seeds are rinsed three to four times with distilled water and then soaked in distilled water for 12 hours at 0 C. This will leach out any unreacted mutagen which would otherwise increase the physiological damage. A running water aftersoak, or changing the water during the aftertreatment time is helpful.

5. The seeds may be planted immediately either in the lab or the field. For field experiments, the maximum amount of seedling damage that will have a good survival in the field is about 30%. The seeds may also be dried and planted at a more convenient time. In this case, an additional 10 - 15% damage can be expected.

These times and concentrations of mutagens must be varied to fit your needs. These are approximations of damage obtained from our results, but they may not hold true with varied conditions.

iPMS - molecular weight - 138.19; density at 0 C - 1.163 g/ml
10 C - 1.152 g/ml
20 C - 1.140 g/ml
30 C - 1.129 g/ml

Hydrolysis half-life 0 C - 38 hours
10 C - 8.5 hours
20 C - 130 minutes
30 C - 35 minutes

EMS - molecular weight - 124.16; density at 5 C - 1.220 g/ml
25 C - 1.203 g/ml

Hydrolysis half-life 0 C - 1716 hours
10 C - 379 hours
20 C - 93 hours
30 C - 26 hours

DES - molecular weight - 154.19; density at 25 C - 1.18 g/ml

Hydrolysis half-life 0 C - 59 hours
10 C - 13 hours
20 C - 3.3 hours
30 C - 1 hour

Phosphate buffer solution:

Dissolve 13.92 g of K_2HPO_4 plus 2.72 g of KH_2PO_4 in distilled water and make up to 1 liter with distilled water.

0.01 M solution of DES:

154.19 molecular weight of DES
.01 molar concentration desired
1.5419 ÷ 1.18 (density of DES at 25 C)

= 1.31 ml made up to 1 liter.

Procedures

Day 1	5:00 PM	Place 1 kg of seed in a 2-liter plastic jar. Fill with distilled water at about 0 C. Place in refrigerator and hold at about 0 C.
	7:00 PM	and 10:00 PM. Pour off water and replace with distilled water at about 0 C. Replace in refrigerator.
Day 2	8:00 AM	Rinse with distilled water at about 0 C. Replace rinse water with a 0.01 M solution of DES at about 0 C. Place in refrigerator.
	2:00 PM	Pour off DES solution and replace with a 0.01 M solution of DES at room temperature.
	4:00 PM	Pour off DES solution and rinse four or five times with distilled water. Replace rinse water with distilled water at about 0 C. Place in refrigerator.
	6:00 PM,	8:00 PM and 10:00 PM. Pour off water and replace with distilled water at about 0 C. Replace in refrigerator.
Day 3	8:00 AM	Pour off water and rinse two or three times with distilled water. Pour off rinse water. Plant immediately or dry seeds for later planting.