Notes for a Laboratory Manual

Short-Cut-Notes (Quick reading before skipping the introduction)

This Manual is intended as a handy REFERENCE near the laboratory table and not as a textbook in a Class-room. Therefore the main body, the practical methodology is written for each typical crop with many duplication among closely related plant species. For example, for mutagenic treatment of barley, some parts are common with the method for rice.

General notes on mutagens, structure of a gene and its actions or functions *etc* are written in the INTRODUNTION part, so that for understanding of genetics of mutation breeding, reading of the introduction is **strongly recommended**. However, if you have enough knowledge and want a quick reference, go to the crop species of your interest or to the one closely related to your material.

In mutant plant screening, reproductive manner of your material is important. Generally speaking, the induced mutant genes are inactivated status, or in other words, they are recessive in nature, so that ONLY HOMOZYGOUS state can express its mutant character, in seed propagated plants (Mendelian segregation in 3 normal to 1 mutant after self pollination) and/or vegetatively propagated plants (knock down of dominant gene by somatic mutation in a heterozygotes). Even within seed propagated crops, for self compatible and self incompatible crops, the strategy should be different. The seeds of barley, wheat and rye look very alike, but **genetic ploidy** and **self compatibility** are very different among these. Barley is a simple diploid, wheat a hexaploid, and rye is self-incompatible.

The major methodologies of crop improvements may reside on cross breeding, where all the chromosomes are mixed together, then separated into the progenies to be screened for better genotype, with occasional crossing over breaking the linkage. Mutation Breeding or Transgenic Breeding (so called GM) can modify only a few numbers of genes, mutation as an inactivation (**subtraction**), and transformation as an introduction of external gene(s) (**addition**). While the former produced more than 2000 cultivars in the world (see IAEA database on internet[1] and/or separate file by E. AMANO[2]) and widely accepted by consumers. The latter, transgenic breeding, is experiencing difficult phase to be accepted by the people. As a technology, transformation is a very important methodology of genetics to clarify the function of a gene. The difficulty might be due to the use of genes UNFAMILLIER for human being. Some efforts are being made in Japan to use genes from materials which are familiar to human beings as food materials so that allergic reaction may not occur for consumers. For the time being, it is still young technology expected to grow.

The basic difference between "mutation" and "transformation" may be in the action of the modified or added gene and its expression. If recessive phenotype or inactivation of a gene is wanted, induced mutation should be the first to be consulted, but if dominant or actually working gene is wanted, transformation, or cross breeding, to introduce the external gene into the crop should be referred.

In some Asian regions, waxy or glutinousness of cereals are highly prized. The waxy character lack a straight chain starch component, called amylose, by inactivation of a gene for amylose synthetic enzyme. Generally, there is only one gene in a genome, so that a single mutation can induce waxy mutant gene in a diploid plant, e.g. rice or barley. It is much easier to induce waxy mutant than transformation (introduction of anti-sense sequence or by gene splitting) or cross breeding, in the latter, genetic background are completely upset by cross pollination. On the other way, that is return form *wx* to *Wx* status, it is almost impossible to retrieve normal amylose synthetic function by induced mutation. If so wanted, only transformation with normal NON-WAXY gene or cross breeding can do the work.

Introduction

In this section, general genetic information is described. You may skip and go to the crop-wise description if you know the fundamentals of genetics.

Methods of crop improvement

1) Cross breeding Mix chromosomes then select the best combination of genes

To create a completely new variety, CROSS BREEDING is the major and the most effective methodology. Mutation or transformation may be good to improve only a few characters leaving most of the other characters specific to the variety unchanged. On the other hand, cross breeding basically upset the entire genotype of the excellent traits of the parental varieties.

Therefore, if you want to improve only a few characters, most of the other characters remain like the parent, mutation or transformation may give good results depending on the purpose, recessive trait or dominantly inherited trait. Mutation may be good for the former and transformation for the latter. Of course, a single cross followed by repeated back-cross may be used to introduce only a few characters from one parent, although it is laborious and takes long period of time.

2) Mutation breeding Subtraction from normal genotype

If recessively inherited character is wanted in otherwise excellent variety, mutation breeding may give good results, like semi-dwarf lodging resistant rice. Reimei (in Japan) or Calrose 76 (in USA) showed good lodging registance in rice cultivation. Linola (in Australia) might be another excellent case of modification of fatty acid spectrum from drying oil into edible oil by changing the major element from linolenic acid (18:3) to linoleic acid (18:2).

When a new cultivation regime, like hybrid rice, are considered, temperature or photoperiod depending male sterility, double pistil, open-hull(but fertile) *etc* (please be noted that these are all recessive characters and will not expressed in the F1 hybrids) may be useful, although they may not be appreciated under ordinary cultivation regime.

This method of selecting homozygous mutant plant in M2 may be applicable where self compatible seed propagation can be expected, however, for crops like most of fruit trees (complicated genotype support the variety traits) like apple, pear, or seedless crops, banana *etc*, or many ornamental flowers, e.g. Canna, orchids, *Chrysanthemum etc* the application needs some considerations.

In case of polyploidy crops, mutation work may not give simple and favorable mutant segregation due to the multiple copies of the related genes. For example, the albino mutant which lost the ability to synthesize chlorophyll are very good indication of the induced mutation in most of diploid species, but it is very seldom in the mutagenized hexaploid bread wheat.

3) Transformation (Gene introduction) Addition of external gene/DNA

In the same way as the mutation work, transformation may give good results in adding the plant by only a few characters leaving most of other genotype remain unchanged. The introduced external gene should have NORMAL function in the new environment, both the function of the gene and the new metabolic environment.

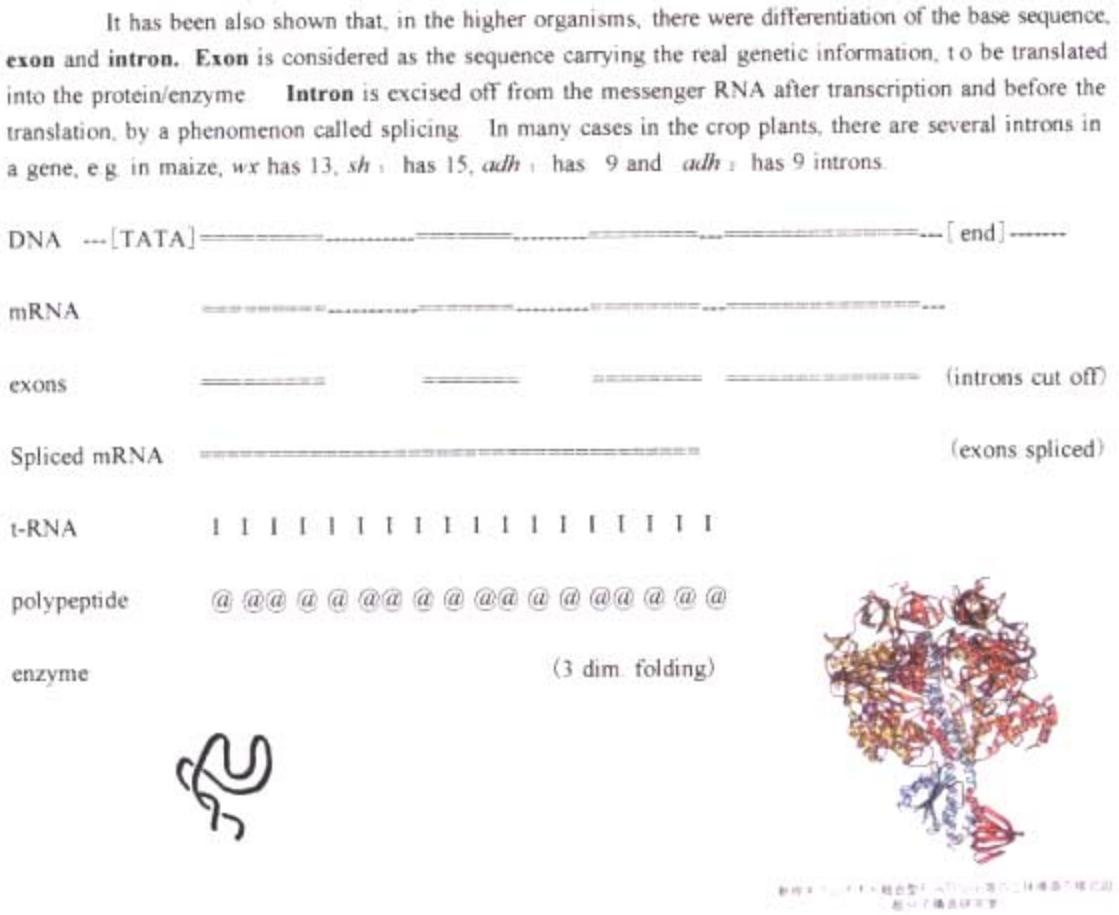
In academic studies of a gene, transformation is a fundamental technology to clarify and to prove the gene function. However, some consumers concern the safety of the output in relation to allergic reaction of human body, which may occur by the gene products the human being had never exposed to. Some people give their effort on the introduction of rather FAMILLIER gene(s) to the human being so that allergic reaction may not occur by eating the new transgenic varieties.

Besides the academic values of the technology, there may be some important ideas in this method; 1) to overcome ploidy difficulty mentioned in mutation work, and 2) introduction of ANTISENSE sequence to overcome multi-copy gene family of the species. Presently it is still growing methodology, but it will be a powerful tool in future crop improvement.

Gene structure

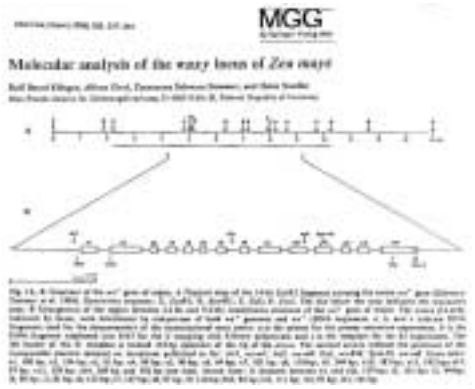
Intron and exon

In describing the activities of a gene, in terms of mutation, its structure is the fundamental. Gene had been considered to locate on chromosome like a rosary or string of pearls. But recent DNA analyses strongly suggest a structure of sentences written in only four letters, A, T, G and C and differentiated into exons and introns within a gene. Each gene generally composed of 2000 to 3000 or more base pairs (or letters). Exons carry genetically meaningful information. Introns are connecting base sequences which will be finally removed from mRNA by splicing. The spliced exons will be translated into proteins by function of tRNAs. The protein will get matured to have 3-dimentional structure to be a functioning enzyme molecule. Following figure[3] is an example of an enzyme protein.



This scheme model suggest that if all the letters, or at least spliced exons are in normal condition, it is a normal or functioning gene, but if there is a modification in the letter, the gene may lose the function or in other words, the gene will be get into inactivated condition, the mutated gene. Some ionizing radiations, especially high LET radiations, e.g. reactor generated neutrons or accelerated ion beams, may introduce deletion of a few bases. Chemical mutagens, e.g. EMS or dES may introduce substitution of base (or letter). Recent studies presented an explanation on the callus culture mutation, as activation of retro-transposon, which might be dormant or inactive in ordinary growth or reproduction conditions. The resulting mutant phenotype may be used as stable genetic resources.

The following figures illustrate example of maize Waxy gene reported in a journal, MGG in 1986 by R. B. Kloesgen et al[4].



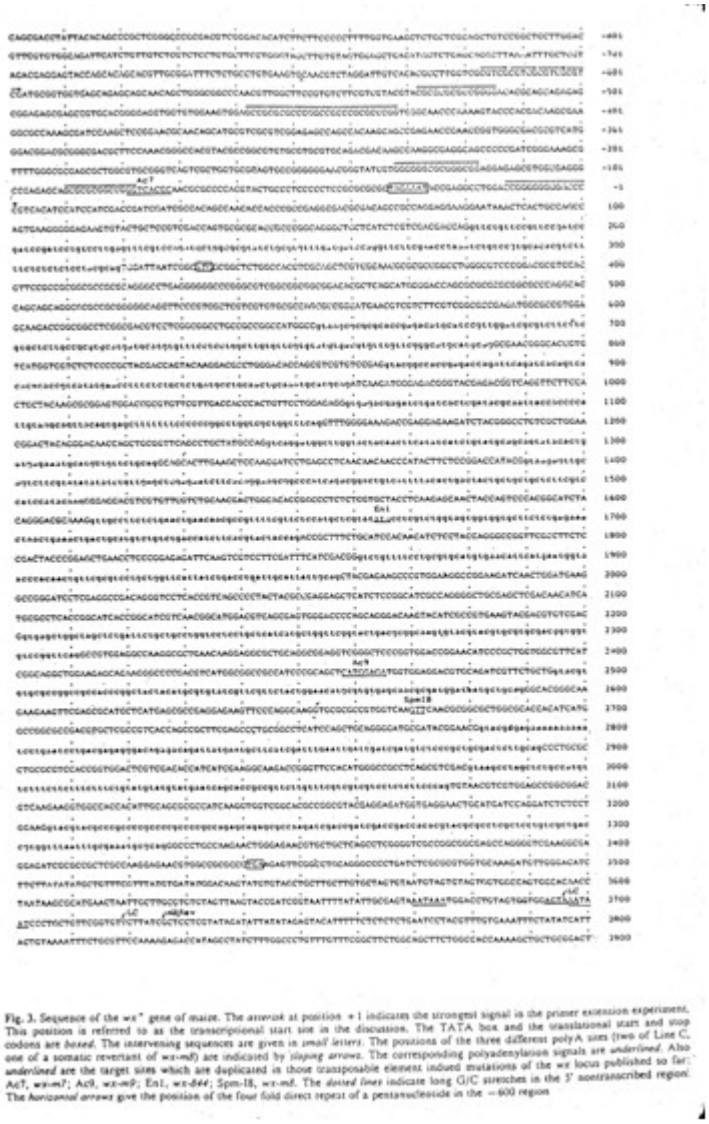


Fig. 3. Sequence of the *wx*$^+$ gene of maize. The *arrowhead* at position +1 indicates the strongest signal in the primer extension experiment. This position is referred to as the transcriptional start site in the discussion. The TATA box and the translational start and stop codons are *boxed*. The intervening sequences are given in *small letters*. The positions of the three different polyA sites (two of Line C, one of a somatic revertant of *wx-m8*) are indicated by *sloping arrows*. The corresponding polyadenylation signals are *underlined*. Also *underlined* are the target sites which are duplicated in those transposable element induced mutations of the *wx* locus published so far: Ac7, *wx-m7*; Ac9, *wx-m9*; En1, *wx-B44*; Spm-18, *wx-m8*. The *dotted lines* indicate long G/C stretches in the 5' nontranscribed region. The *horizontal arrows* give the position of the four fold direct repeat of a pentanucleotide in the −600 region

Mutagens

Physical agent

Here in this laboratory manual, mainly X-rays and Gamma-rays will be considered. Ion beams and neutrons with various energy may be also utilized in specific purposes, but penetration or dosimetry may be beyond the topics described here, although they are very important elements in irradiation work.

With X-rays, penetration through the materials depends on the high voltage used to operate. In ordinary enveloped seeds or cuttings, the thickness should be less than a few centimeters, when 100kV to 250kV is used. The sample thickness may also be important not only for penetration but also in terms of the distance from the radiation source. The X-ray tube is active only the high voltage is turned ON.

Gamma-rays from 137-Cs (660keV) or 60-Co (1.17MeV+1.33MeV) may penetrate even glass tissue culture flasks. In case of these Radio Isotope irradiation sources, the gamma-rays are continuously radiated from the source, so that the safety precautions should be the first priority for the operating personnel.

For botanical staff members, generally dosimetry may be left to the operator/physics staff. Basically, so called secondary electron equilibrium, is important. The necessary condition is easily achieved by putting 3 to 5 mm acryl resin-plate or other comparable plastic plate on the irradiated sample.

The irradiation dose should be tested before formal irradiation. In this preliminary exposure experiments, number of sample per dose-class may not be large, some 20 seeds per dose for example, but the dose range should be extended from very weak to almost kill all, in 10 classes or so. About 20% reduction dose for the growth (seedling height or

reduction of the grown (fresh/dry) weight) may be suggested to use (see the example in RICE section). For radiation biology work, LD50 (50% lethal dose) is a very clear index, but for mutant induction, it is too high to give good growth/results.

Chemical mutagens

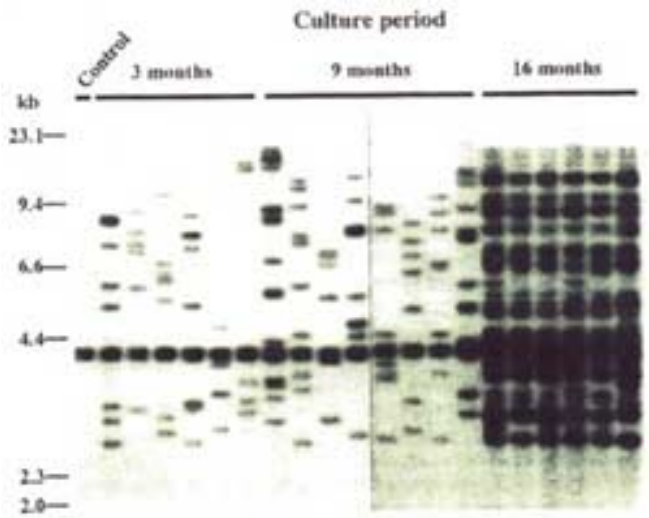
Gamma-rays from 60-Co will be reduced by the HALF-LIFE of five years, which may not be the concerned factor of most of biological staff members (except executive and dosimetry staff). However, with chemical mutagens even biological staff must handle by themselves in their laboratory. The same half life problem of the agent arises for the chemical mutagen. Compared to 60-Co, the half life of effective elements of most of chemical mutagens in the water solution is very short, matter of a few hours to several hours. Mutagenic agent, physical or chemical, must be carefully handled because carcinogenicity is suggested almost all the mutagens. In case of chemical agents, hydrolysis, alkali- or acid-disintegration are possible before discarding the wastes, solution or wiped papers *etc*. The treated materials, seeds *etc*, should also be carefully handled.

Ethyl methanesulfonate may be suggested as most effective and easy to handle in storage (refrigeration), use (1% or less water solution) and waste disposal after alkali-disintegration. Once there were discussions on the chromosome breakage ability of EMS, but as far as pure water (distilled and deionized water, and not with any buffering agent) is used, it induces good point mutations including leaky or mis-sense mutations[5]. Refrigeration (not frozen) storage is suggested for EMS. The cool bial of EMS should not be opened, as the dew drops may hydrolyze and destroy the agent. So, EMS should be stored in a refrigerator in an air-tight container with ample amount of Silica-gel drying agent (refer to PictureWord file "14EMS"). Take out from refrigerator a few hours before the use, to have temperature balancing with the room-temperature. Then, open the bottle. Never try mouth pipetting. If good pipetter is not at hand, a (plastic) syringe connected to glass pipette by short tubing can a good substitute to measure the agent.

Biological treatment

Remote hybrids often produce aberrant phenotypes in the progeny. Aneuploidy plants (chromosome numbers deviated from euploidy (diploid, tetrapoid etc.)) will give chromosome aberrant plant which may show aberrant phenotype. This special phenomena are often used in developing aneuploidy system e.g. in wheat.

Apart from such very special cases, aberrant character which appeared after callus induction, culture and plant regeneration experiment (including anther cultures) are often reported as somaclonal variations. Recent studies explain the phenomena as activation of otherwise stable RETRO-TRANSPOSON (Hirochika *etc*[6, 7, 8]). When the cells got special kind of stress like dedifferentiation, e.g. calls induction, the retro-transposon is activated and produce copies of it as messenger RNA, which is then used in synthesizing DNA sequence(s) ("retro" way!) that will act like transposon. The period of callus culture increases the number of DNA copies.



In this Figure, Control is ordinary japonica rice variety, having a stable single band, suggesting highly stable status under ordinary condition. After three months of callus culture multiplicated DNA (retro-transposons) had increased significantly. Nine months and 16 months gave too many DNAs which might be inserted at too many sites inducing too many gene inactivations or mutations. For further information, see attached report "Manila2.DOC" including references by HIROCHIKA.

For mutagenic treatment, only a few months may be suggested. Too long callus culture may produce only albino regenerated plants perhaps due to too much transposons produced. Further studies suggest that the mutant thus induced, and the causing retro-transposons are stable under ordinary field agriculture. One possible difference from the radiation induced mutation, in which complete randomness can be expected, may be in the preference or distortion by the biological system. For example, in some mutants, e.g. rolled leaf in rice, all the members of the family often show the same mutant character. If it were dominant mutation, there must be some normal plants segregated to 3 dominant mutants to 1 normal. The reason of such abnormal segregation still remains to be studied. Otherwise, mutants thus developed and detected will be used as stable mutant line. Please be noted that other "transposon" mutants may revert to original non-mutant phenotype relatively frequently.

The regenerated plant may be in R0 generation as the researchers in this field call it. R0 is comparable to M1 of irradiated or chemically treated materials, that is heterozygous generation regarding the mutated gene(s).

It should be noted that "generation designation is different in M (mutagenized) and R (regenerated).

Mutation is caused by insertion of the (retro-) transposon into existing gene(s). Perhaps complete inactivation of the inserted gene may be expected by insertion of the large sequence of transposon. Screening of mutant should be made in the R1, *i.e.* M2 comparable generation.

Dominant / recessive

This is to repeat the description of when and where the technique should be applied to improve crop plants.

Cross breeding : Effective for all STABLE genes

This is a fundamental methodology to improve crop plants. Recent tissue culture techniques may be useful in remote hybrid making through embryo rescuing. In some cases, cell fusion may be used to produce somatic hybrid through protoplast. The basic pollination hybrid is to make chromosome mixture, and then screening of favorable genotype (phenotype) is the fundamental procedure to create new varieties.

Mutation : Effective only for recessive characters

When the gene inactivation or its phenotype (recessively inherited character) is required, mutation induction will be a simple, low cost and easy way to get good results. However, where gene exist in multicopies, like polyploid plants, other methodology must be consulted also.

Transformation : Effective only for dominant genes

When fully active gene or a new and active gene is wanted this transformation method may give good results. Some consumers concern the safety of the newly introduced materials (protein as a gene product), but the technique itself is very powerful tools in academic research work on the gene. Some people are trying to introduce genes well accustomed to us, human being, for long time in certain food materials. For example, only moving one gene from one bean species to other bean species may not give serious allergic reaction for human, but it was effective to reduce insect damage in azuki bean.

Anti-sense DNA sequence introduction may have another very interesting power, in some expression SUPER DOMINANT inheritance expressing the RECESSIVE character. This may be a very good study item for the future molecular work.

Extra-information

Size of chimerism (mutant sector)

Besides the gene genetics, structure of plant tissues and reproductive characteristics needs special attention in mutant induction and screening. When a plant/seed is mutagenized, e.g. in rice seed/embryo, perhaps mutation will occur

singular in a nucleus of a cell, in other words, the mutant gene will be hetero-zygously present. After self pollination of such heterozygotes, Mendelian segregation will produce homozygous recessive mutant plants in about 25% of the progeny family. However, suppose a cucumber or maize, seeds of which had been treated by effective mutagen.

The embryo in the treated seed had already in a multi-cellular condition. One cell may have a mutated nucleus, but the next or the neighboring cells may not. Such heterogeneous cell population form chimerism in the M1 plant. Then, it may highly be possible the male pollen of a maize formed on the top of the plant might come from different chimeric mutant tissues from the lower female flower/cobs of the same plant. If such occurs, self pollination within the same plant is not really the self pollination in the case of rice, i.e. within the heterozygous tissues, to have Mendelian segregation. It may sound strange, but it may be visualized if you use a leaf color heterozygotes as the treatment material, e.g. *Yg*2/*yg*2 in maize. If you have many M1 plants you may see chimerism like the Photos (maize male tassel albino mutant tissue) shown here.

In vegetatively propagated plants, visualization may be difficult, but the genetics and idea are the same. In these cases, non-chimeric or so-called solid mutant tissue/sport may be available after a few vegetative generations, in M1V3, M1V4 or later

Brushing Up a Mutant

Induced mutant line very often contains "unfavorable simultaneous mutant gene(s)", which very often degrades the mutant quality to be a practical variety. Too hasten evaluation of the mutant may also evaluating the MINUS factors of these extra mutant genes. Unfavorable simultaneously induced mutant gene(s) may be cleaned up by backcrossing and re-segregation of the intended mutant phenotype again. Very often, a single backcross to the original line may be good enough to recover the original seed fertility, for example. Backcross to the original line may not mean cross breeding, as almost all the background genotype is maintained as the original line.

In case of vegetatively propagated materials, cleaning by backcross can not be applied in most of the cases. If so, try to induce mutants as many as possible, then screen a clone with the best performance. One basic suggestion for the both types of the crop, apply minimum of the dosage, physical, or chemical mutagens. It may require large M1 and M2 population. But please understand that the frequency of unfavorable mutant gene is also depend on the mutagen dose applied, the higher the more.

> Suppose about 10,000 genes are active in a plant, e.g. in rice, where only one *Wx* gene is in a genome and showing about 1/1000 mutation frequency, if we select a *wx* mutant, the other genes may have the same frequency in mutational change, that is 1/1000 x 10,000 = about 10 simultaneous mutant genes. Practically of the ten such mutant genes, perhaps only a few of them may be included in the selected mutant line. Then the performance test of the mutant may also include this extra mutant genes. If these genes were separately located on different chromosomes, single back cross may be effective to exclude them. Sometimes, such simultaneously induced second mutation give a bonus character, e.g. BYMV resistance in an early maturing mutant line found in Japan, or the case of salt tolerance of a malting barley 'Golden Promise' in England. But such characters should be screened as the major purpose of the mutation breeding.

Where to search

In this manual, crop plants are grouped at first by the reproductive pattern, seed propagated or vegetatively propagated. Further characterization will be described in each crop group or species.

Each item may have following components.

Groups of plants

For mutant induction and screening, reproductive pattern and chimerism in the treated material is very important. The chimerism may be visualized by use of appropriate genotype material, but before such information is available, safe strategies will be suggested in the practical description section. For example, a big head of Solghum may be composed of a few chimeric sectors when a seed is treated. Before the chimeric development is visualized, it may be safe to say that the mutant sectors may be formed rather in radial pattern around the plant axis. In rice, the central axis panicle may have chimeric mosaic structure, but most of the panicles developed later usually segregate 1 mutant and 3 normals. Such information will be obtained during the experimental procedure.

Although modification in accordance with the chimeric structure of the plant is needed, basic seed numbers in one unit or panicle/ear/cob may be important in mutant screening. Following lists may suggest some of such groupings. Each crop species should be separated further by polyploidy or other factors.

The number of seeds per panicle/cob/ear/fruit or putative chimeric unit is important in expecting the Mendelian segregation.

Mutant selection may depend on the purposes of the breeding, sometimes surviving screening applied to pooled M2 materials may be very effective, but especially for academic purposes where homozygotes is difficult to survive, harvesting of heterozygotes are also important, then M1 panicle/spike/cob family system should be considered.

1.	Self pollinating crops		
1-1.	Diploid		
1-1-1	Very many seeds per unit	more than 300	Millet, Solghum
1-1-2	Many seeds per unit	more than 100	Rice, tomato, sesame
1-1-3	Good seeds per unit	more than 50	Mung-bean,
1-1-4	Small number per unit	around 10	barley, soybean, peas
1-2.	Polyploid	macaroni wheat (4x), bread wheat (6x), triticale(rye-wheat : 6x or 8x)	
2.	Outcrossing crops		
2-1.	Self-compatible		
2-1-1	Very many seeds per unit	more than 1000	Orchids
2-1-2	Very many seeds per unit	more than 100	Cucumbers, maize, melons
2-1-2	many seeds per chimeric unit	more than 50	maize (in terms of chimerism)
2-2.	Self-imcompatible		
2-2-1	Very many seeds per unit	more than 1000	certain orchids
2-2-2	Small number of seeds per unit		Rye, buckwheat, radish,

3. Vegetatively propagated

3-1. Herbaceous : Chrysanthemum, Canna, some orchids

3-2. Arboreals : Rose, Fruit trees (incl. Oranges (partheno-carpy?))

To develop a new variety, cross breeding is effective, but with established varieties

Cutting, grafting etc are used not to disturb the variety genotype.

4. Seedless plants

Banana, (*Lycoris radiata* Herb.), Seedless grape, Some oranges

Mutation or transformation may be the only method to modify genotype.

5. Polyploidy and other plants

Sugar cane,

High chromosome number and vegetative reproduction, also allows aneuploidy.

Wheat

Very important cereal, but hexaploid species giving difficulty in mutation breeding.

Orange group

Seeds formed in fruit is very often developed from percenogenic maternal tissue so that cross

breeding is not so effective method to improve.

6. Other organisms

Diploid/haploid generation and mushroom/hypha stages may need some extra strategies but basic genetics will be applicable.

6-1. Mushrooms

6-2. Yeast, Molds and other fermentation organisms

Extra Suggestions

Visualization of mutant tissue

If you are going to work on your materials for more than a few years, keep an appropriate color mutant and produce a heterozygote to study the chimerism pattern of your material. In case of rice, viable yellow green (chlorina) mutant may be used. If heterozygotes are available in a segregating family, it may be used also. In case of self incompatible buckwheat, seeds may be considered to have chlorophyll genes accumulated as heterozygotes in most of them, as they very often give white or yellow sectors after mutagen treatment like a picture shown here.

Somatic mutation sectors appear after mutagen treatment, according to its growth pattern. In maize, mutant sectors become countable from leaf 3 after seed treatment. Generally, mutated cell might not be disturbed its growth, giving very clear sector against normal tissues and showing typical growth pattern of the tissues (stripes in cereal plants, and fan-shaped sector in buckwheat).

Visualization of chimera will be important in melon/cucumber group. After mutagen treatment, usually tertiary vine developed after pruning twice, may be free of chimera, so that pollination between male and female flowers on the same tertiary vine may give mutant segregation in the seeds thus obtained.

Selection method (Visualization of mutant segregation)

Take rice or barley as an example. The genotype is composed of two set of chromosomes, one from pollen parent and the other from maternal egg cell. Each component has corresponding genes. Induced mutation will occur only one of those corresponding pair. Almost all the induced mutant genes are broken or inactivated (recessive) state. So, in M1 generation, somehow the normal gene may show non-mutant character. In order to find and screen the mutant, homozygous plant must be produced. This is the fundamental Mendelian segregation.

♀ \ ♂	Male zygotes	
Female zygotes	A	a
A	AA	Aa
a	Aa	aa

1AA : 2Aa : 1aa
Normal homo Heterozygotes Mutant homo
Normal Phenotype 3 : 1 Mutant Phenotype

M2 planting pattern

In most of seed propagated crops may segregate in M2 generation at the rate of one mutant to three normally looking plants as illustrated above. This suggests that if ten M2 plants out of one mutational unit (panicle or ear in cereals) two mutants may appear among the sister plants. About ten to 15 plants may be suggested as the segregating family size. In the Institute of Radiation Breeding in Ohmiya Town in Japan, they used 15 plants for a M1 panicle family for rice. In Fukui, after integrating special machine transplanter, 14 plants for a panicle family were set (see attached pictures). Duplicate or triplicate appearance of mutant phenotype assures us that they are the induced mutants.

After experiencing the segregation of mutants, each researcher may develop and proceed his/her own system depending on the purpose of the experiment. If lethal mutation like albino or disease susceptible mutant are the target of the academic studies, this panicle/ear family planting is a necessity to keep the viable heterozygotes in order to maintain the mutant gene.
Because this panicle/ear family planting needs both field area and sowing/transplanting labor, if the mutagenesis is integrated in the ordinary plant breeding program, one seed (or a few seeds) from one panicle/ear may be adopted, because, perhaps one mutant plant may be good enough to start up the improvement.

Survival Screening
In an extreme case, mass sowing or planting and selection of a few survivors may be effective e.g. cold tolerance, disease resistance, insect and other pest resistances. Planning of the screening system of only a few plants out of 100,000 M2 plants should be considered.
In microbial (bacterial) genetics, this is an ordinary method of screening, but in higher plants, even a breeder may feel some guilty to discard almost all the plants.

Stress application
Not only in the survival screening, but in some screening a light stress may be used in many phases of screening, e.g. day length or lodging. Selection by yield and other selection pressures may be another human factor of stress placing.
In case of survival screening, it may be natural to place all the M2 seeds under the stress pressure, but in case of lighter pressure, it may be advised that repeated screening with the light pressure may concentrate the mutant gene(s).

Basic studies
The purpose of plant breeding is to obtain and disseminate favorable varieties to the farmer, hobbyist or other users. For this purpose, any resources may be used, and even contamination may give good results. In some cases, the breeding may be described as a kind of fine art, deeply depending on the breeder's personal sense. However, to become a reproducible technology, the method should be scientifically sound. The difference between science and fine art may be in that, in science there may be no room for human being, but in the fine art, the results is the creative work of a human being. Gregol Mendel made clear the rule of the segregation in the nature. But he did not set the ratio, but the NATURE set the ratio 3 to 1. In other words, even without Mendel, dominant and recessive will segregate in 3 to 1 ratio. (Actually the segregation rule was re-discovered 35 years later by three scholars in 1900.)
It may be better not to mix the science with practical application of science, but scientific back ground may be a good guaranty of reproducibility of the next good variety.

Additional Information (See the following websites)

Radiation sensitivity (Amano's collection) http://www.fnca.jp/english/mb/mbm/pdf/1_radslst.pdf

Excel file http://www.fnca.jp/english/mb/mbm/pdf/2_radiation_sensitvty.xls

Mutant varieties in the World (based on IAEA publications)
http://www.fnca.jp/english/mb/mbm/pdf/3_mutant_varieties.pdf
Excel file http://www.fnca.jp/english/mb/mbm/pdf/4_mutant_varieties.xls

This list is the output from the Mutant Cultivar Database by E.A., based on the IAEA publications. Key-word search was applied to analyze the data.
Please note that this is a personally written table and there may be problems in the plant materials (use of hybrid seeds *etc*.), or reaction of the crop plants (radiation against *Zea mays* (maize) *etc*.). Further more, in the USA, the use of mutant in cross breeding are often "Not Reported to IAEA" so that some mutant cultivars are missing.
However, still some trends may be seen from this table.

(NOVAR44withNote.doc : by Microsoft Word v00)

Plant cultivars derived from mutation induction or the use of induced mutants in cross breeding. Data from Mutation Breeding Review (No.3, 1986,3) and Mutation Breeding Newsletter (up to No.44, 1999,4) were analysed by <Keyword Searching> (by E.Amano).

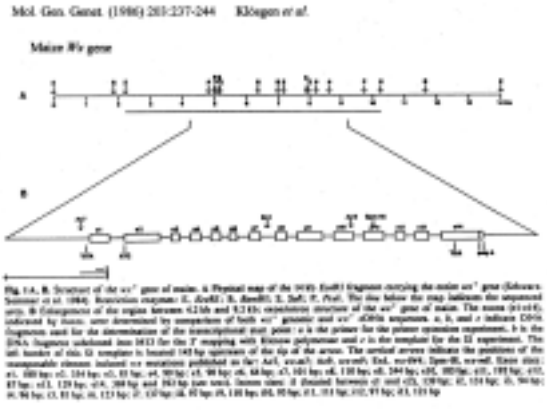
Country	No. of var.	Mutant used in cross	Crops															Mutagen*					Note**
			Cereals					Legume			Ornamental			Fruit		Other							
			Oryza	Hord.	Triti.	Zea	Other	Glyc.	Phas.	Other	Chrys.	Flower	Leaf	Arbo.	Herb.	Fibre	Other	X	G	N.	Other rad.	Chem.	
China	314	73	117	1	81	23	4	23	0	26	0	7	0	3	2	8	22	9	218	17	2	3	las 14
India	253	42	35	14	4	0	5	0	3	35	46	57	0	1	9	13	31	28	145	7	5	26	chr 8
Russia(USSR)	209	72	6	28	36	12	12	9	4	30	17	7	1	9	4	4	30	1	56	0	5	86	las 1
Netherland	176	1	0	1	0	0	0	0	0	0	80	93	0	0	0	0	2	153	17	5	1	3	
Germany	140	48	0	46	2	0	2	1	2	1	34	45	0	1	0	0	6	57	29	0	1	5	
Japan	120	43	46	8	2	0	1	6	0	1	14	9	8	4	4	3	14	2	74	0	2	4	chr 16
USA	105	51	23	11	3	0	11	0	6	1	1	20	8	2	0	0	19	10	29	17	2	6	
France	42	14	5	15	1	0	0	0	0	0	0	14	0	7	0	0	0	0	24	0	0	4	chr 1
Italy	35	9	1	0	15	0	0	0	2	6	0	0	0	5	4	0	2	7	8	5	0	7	
Czech(CSSR)	34	25	0	27	0	3	0	1	1	1	0	1	0	0	0	0	0	2	3	0	0	2	chr 3
Brazil	34	3	27	0	2	0	0	0	2	0	3	0	0	0	0	0	0	0	31	0	0	0	chr 26
UK	33	31	0	32	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	
Bulgaria	30	6	0	4	6	8	0	3	0	1	1	1	0	1	3	0	3	2	14	0	0	1	
Poland	30	17	0	1	0	0	0	0	1	21	6	1	0	0	0	0	1	4	9	0	0	0	
Cote d'Ivoire	26	17	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	chr 9
Guyana	26	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	chr 26
Sweden	26	20	0	20	0	0	0	0	0	1	0	0	0	0	0	0	5	6	0	0	0	0	
Pakistan	24	0	4	0	4	0	0	0	1	12	0	0	0	0	0	2	0	0	18	1	0	2	
Vietnam	24	6	14	0	0	2	0	4	0	1	0	0	0	2	0	0	1	0	9	0	0	12	
Belgium	22	1	0	1	0	0	0	0	0	1	7	8	3	0	0	0	2	16	10	0	0	2	
Canada	22	12	0	2	0	0	0	0	1	0	0	4	0	8	0	2	4	7	2	1	1	2	
Denmark	22	20	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	
Iraq	22	2	2	7	6	0	0	0	0	2	0	0	0	0	0	0	5	0	17	3	0	0	
Austria	20	17	0	12	6	0	0	0	0	1	0	0	0	1	0	0	0	1	2	0	0	0	
Bangladesh	16	3	3	0	0	0	0	0	0	3	0	0	0	0	1	3	6	2	6	0	0	2	
Finland	11	6	0	4	1	0	6	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	
Indonesia	11	1	6	0	0	0	0	3	0	1	0	0	0	0	0	0	1	1	9	0	0	0	
Korea	11	2	2	1	0	0	0	2	0	0	0	0	0	0	0	0	6	4	1	2	0	3	
Thailand	9	1	4	0	0	0	0	1	0	0	2	1	0	1	0	0	0	0	7	1	0	0	
Australia	7	3	0	0	0	0	2	1	0	4	0	0	0	0	0	0	0	0	0	0	0	4	
Argentine	6	0	0	0	1	0	0	0	0	2	0	0	0	3	0	0	0	3	2	0	1	0	
Estonia	6	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	
Hungary	6	1	3	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	2	2	0	1	
Cameroon	4	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	chr 3
Myanmar	4	1	2	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	3	0	0	0	
Burkina Faso	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Costa Rica	3	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	0	
Egypt	3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	0	3	0	0	0	
Mongolia	3	2	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Nigeria	3	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Philippines	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	
Sri Lanka	3	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	3	0	0	0	
Chile	2	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Greece	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	
Kenya	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	2	0	0	0	
Norway	2	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Senegal	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	chr 2
Ukraine	2	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
Algeria	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Guinea Bissau	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	chr 1
Madagascar	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Portugal	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Switzerland	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
Togo	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
*** Total(cumultv)	1917	564	372	267	177	49	44	55	24	156	212	269	20	48	27	36	164	318	810	64	18	178	chr 95
**** Total(real)	1903	564	341	265	177	49	43	58	24	156	211	265	20	48	27	36	152	318	810	64	18	176	chr 64

*: Mutagen : X :X-rays, G : Gamma-rays, N : Neutrons, Other rad . : In early phase data, some records were without details. Beta rays, electron beam etc. are included here. Chem. : Chemical mutagen.

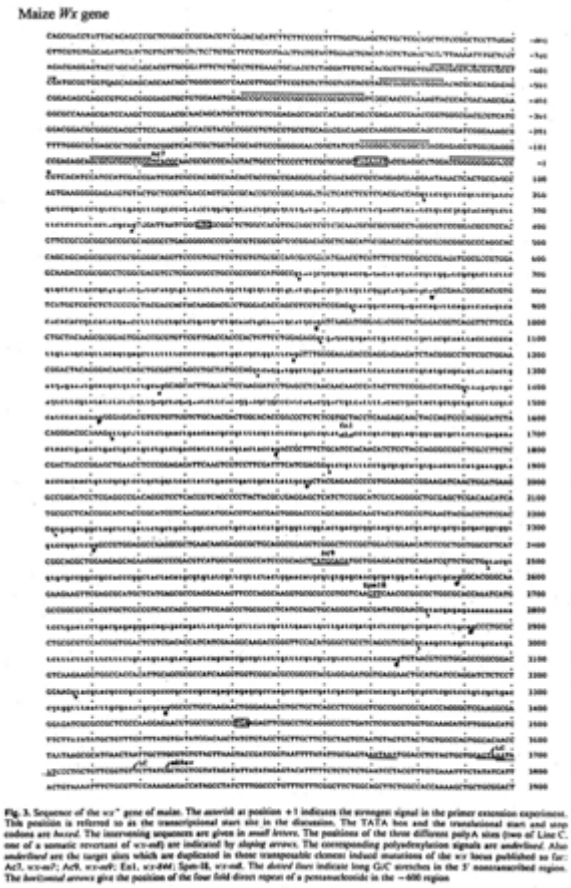
**: chr : Chronic irradiation, las : Laser irradiation (These are included in G and Other rad. respectively).

***: cumultv : Cumulative sum of each column (including duplication among countries).

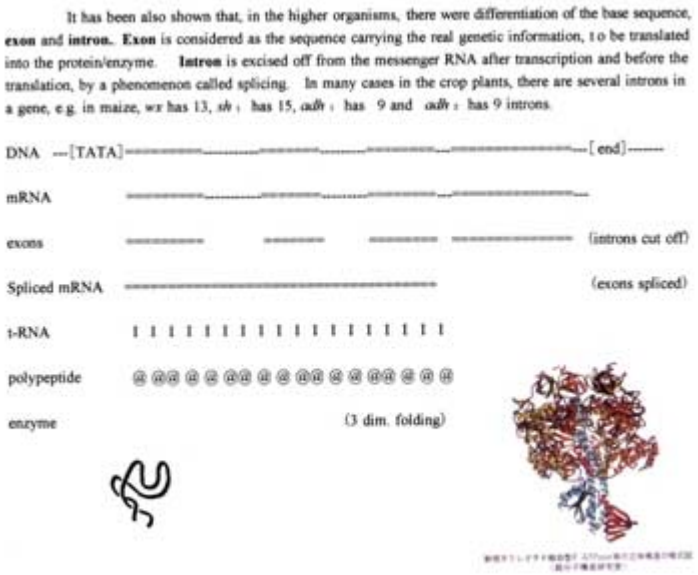
****: real : Actual biological numbers (without duplication).



Structure of higher plants has been reported in 1980's, to have exons (meaningful part) and introns in the gene, the locus on DNA chain.



The gene locus, or its genetic information, is written in the four letters A, T, G and C. In this picture, small letters (lower case letters) mean intron and capital letters indicate the exons.



Information written on DNA is transcribed to messenger RNA, then introns are cut off and exons are spliced together to be translated to proteins or enzymes by the function of transfer RNA which carry amino-acids to the protein synthesis sites. Proteins synthesized may look like the right colored figure. The left simplified figure may suggest the pockets for enzymatic function.

First letters	Second letters: U	Second letters: C	Second letters: A	Second letters: G	Third letters
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA nonsense	UGA nonsense	A
	UUG Leu	UCG Ser	UAG nonsense	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Amino-acids are selected and carried by t-RNA by the list of triplet codes. The second and/or third letters may have less meaning, called redundancy.

The gene disturbed or inactivated by mutation is recessively inherited or expressed, as the normal gene which still has function will be appeared as the dominant gene. In the table, normal dominant gene is shown as "A" and the recessive mutant gene as "a".

♀ \ ♂	A	A
A	AA	Aa
a	Aa	Aa

1AA : 2Aa : 1aa
Normal homo Heterozygotes Mutant homo

This is shown in the garden of Mendel Museum in Brunn, Czech Republic.

This might be only for decorative explanation. The purple Pansy at the top left is dominant parent and the right white as recessive. Then F_1 hybrid is purple, expressing the dominant gene. After self pollination, segregation of three purple and one white occur. Heterozygotes segregate again in 3:1 ratio, but homozygotes at the far left (dominant) and far right (recessive) stay as pure line. The Display also shows up to F3 Generation. The lady at the right is an IAEA trainee from Portugal.

1. Rice

1) How to prepare starting materials

The breeding method using induced mutant(s) will improve only a few characters, e.g. semi-dwarf (for lodging resistance) or early flowering or waxy starch, leaving other characters unchanged at all. So, this method may be applied best to a local varieties. Many kinds of mutant characters will be found during screening procedures. Therefore, it may be necessary to have clear objectives of the expected improvement. Otherwise, many newly induced characters may draw interests of the observer, finally giving too much work on unnecessary characters.

To prepare the starting population (materials), if possible, isolation by paper bagging *etc* may be strongly suggested. Use of hybrid seeds in mutagenesis is not a good idea, unless specific purpose of chromosomal translocation is intended.

> "Hybridization enlarges the possibility of mutation by enlarged number of target gene" is erroneous, as almost all the recessive mutant gene is already inactivated so that further mutation will not give mutant gene at all (only giving double mutant).
>
> Furthermore, hybrid of mutant gene wx-a and mutant gene wx-b may produce completely normal Wx gene by normal (ordinary) intra-cistronic recombination even without mutagen treatment, at the rate of one in 1,000 or so, depending on the distance between wx-a and wx-b. Such result may be mistaken as a dominant mutation.

Keep some original seeds for future backcross.

Use some seeds for treatment control to examine the treatment dose and other procedures. This may be important when you send out seeds to other institution for treatment/irradiation. A small control seeds to examine the transportation condition is important sometime.

2) Mutagens suggested

This depends on the purpose of the work.

If any kind of mutation is accepted for crop improvement, irradiation by X-rays or gamma-rays, or if you have a small chemical laboratory, chemical mutagen e.g. EMS may give good results. In case of Sodium Azide[1], please consult to appropriate literature for necessary pH and/or exact growth stage to apply.

If **Deletion mutation** is wanted, intended to extract a specific mutant gene, ion beam, reactor neutrons or other appropriate high-LET radiation is suggested[2]. In some radiation, e.g. fast and/or thermal neutrons, small but appreciable amount of radioactivity will be induced by irradiation. Special care should be taken in practical handling and also to meet regal restrictions.

If **intermediate or leaky type mutation** is wanted, chemical mutagen, e.g. EMS, which may cause base-pair substitution mutation may be suggested[3, 4].

The idea will be extended to other special type mutation, like **temperature dependent mutations**[5], which may be explained by temperature dependent 3-dimentional folding of protein, the gene product, although it is not proved yet. A comparable rice mutant was also detected in Vietnam, but the critical temperature was a little different from that of Japanese. If the explanation proved to be true, chemical mutagens to induce base-pair substitution may yield such mutation as temperature dependent mutations, perhaps.

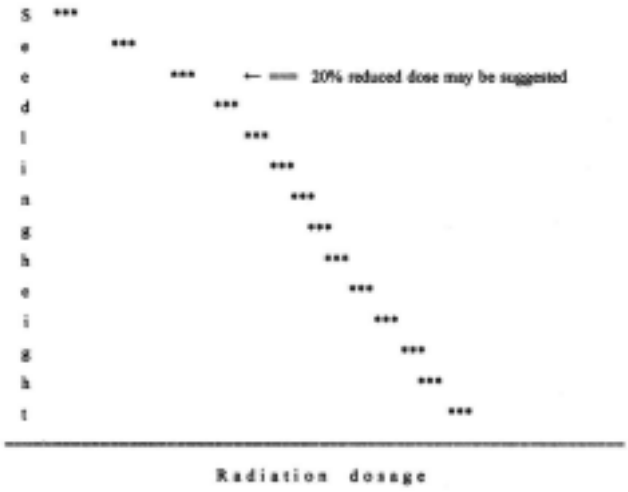
3) How to decide treatment dose

This may be the very important procedure in the mutagenesis experiment. The sensitivity tests are different for radiations and chemical mutagens, the latter is usually applied as water solution, needing one more step of preliminary test.

Irradiation

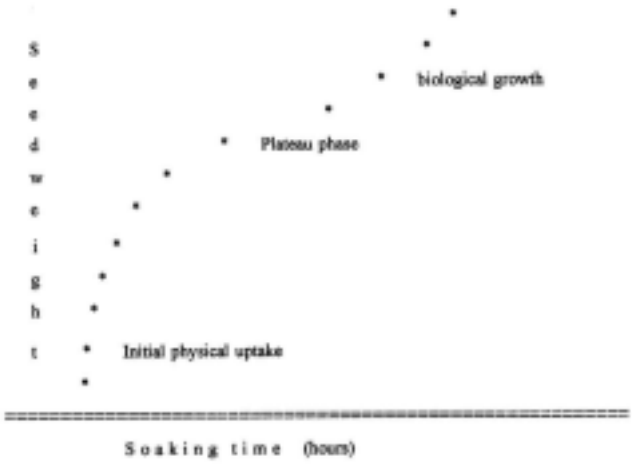
In case of irradiation treatment, sensitivity differentiation is reported in some materials perhaps by a few sensitivity genes. Generally speaking, long grained indica rice is more resistant to radiations than short grained japonica varieties.

To decide appropriate dose, seedling height measurements from 100 Gy to 700 Gy, to cover almost no effect to heavily damaged dose should be tested at first, in 5 or more dose classes with up to 20 seeds each. About 20% reduced dose may be suggested as recommendable dose, where M1 seed set is not much reduced. For radio-sensitivity comparisons, LD-50 (50 %lethal dose) or RD-50 (50 % Reduction dose) are often used as critical values. However, for mutant induction work, such doses are too high for better seed fertility and less accompanied mutation.



About 20 % reducing dose for seedling height may be recommended.
For chemical mutagens, another curve to show the water imbibition is important to decide the treatment.

Dry seeds show very fast water uptake in the initial several minutes, then it reaches to a plateau, where chemical treatment (soaking) is suggested. The strength (concentration) test of the water solution may be comparable to the radiation dose test shown above.



Ethyl methanesulfonate (EMS) treatment was very effective in rice. One example of a recipe may be as follows :

Presoaking in distilled water for two hours.
Soaking treatment in 1 % EMS at 25℃ for 5 hours while shaking gently.
Post wash a few times then keep in distilled water over night.
After final washing, seeds may be sown to a seedling bed.
All paper and glass wares and rinsing water used in the EMS treatment should be put into a bucket of appropriate NaOH solution (1 % or so) to hydrolyze the carcinogenic mutagen.

4) How to grow and harvest the M1 generation

After the mutagen treatment, rice seeds of M1 generation may be planted in field according to the local procedure. Genetically speaking, each treated seeds should be a separate unit in terms of mutagenesis, so one seedling per hill/hole may be suggested. If rice transplanter machine is employed, plan well for the number of plant per hill. If one plant per hill can not be attained, use much more seeds for mutagen treatment and harvest one panicle per hill, neglecting other seeds in the same hill. (This worked well in the author's experiment.) The purpose of planting M1 generation is to advance the generation to M2. So, plants should be maintained to obtain good seeds by application of various pest controlling procedures. Do not attempt to put any kind of screening in the M1 generation, because the induced mutant gene (recessive) is not fully expressed at this stage. Occasionally chlorophyll mutant stripe may be observed in few plants among thousands of M1 plants. This may be a double mutation at the same chlorophyll gene locus. Most of it will be expressed only on some leaves, but it may be a good indication of successful mutagenesis treatment.

> Suppose a mutation frequency of a gene as 0.1 % or one in 1000, a possibility of two mutations in the paternal and maternal gene at the same time will be 0.1 % x 0.1 % = one in 1,000,000. Leaf primodial cells in a seed embryo may amount to 1000 or more. Thus this double mutant sector may be visualized in 1,000,000 treated cell population or a few stripes in thousands of plant.

The central panicle may have chimerism, but most of later panicles seem to be practically free from chimerism. Supposing the panicle-wise screening in M2 generation, suggested harvesting system is as follows, each group should carry name tags accordingly :

1) **The First Round Harvest :** Harvest only one panicle per hill, all through the M1 population. In all the harvested materials, don't forget to place name tags in the way available in the local condition, cultivars used, treatment dose, the first round of harvest *etc*. The first round harvest may be used to examine endosperm characters, waxy, sugary, giant embryo *etc*. It may be difficult to keep the seeds separately after seed character examinations as you may need thousands of envelopes, normally looking seeds may be discarded.

2) **The Second Round Harvest :** In the same way, second round harvest should be made again from the beginning of the M1 population. Some of the panicles may genetically duplicate from the First Round Harvest, but if you use the Second Round Harvest in a different category like plant type characters expressed after germination, duplication is not a problem.

3) **The Third Round Harvest :** Because M1 generation planting is laborious work, this 3rd round harvest may be suggested to preserve for future work. Store the panicles in dry and cool condition for possible future use. In the author's experiences, out of such stored materials, another research institution found a TGMS mutant, and a few recessive tall mutant with *sd*1 (Reimei) background, both may be useful in HYBRID RICE system.

5) How to screen the wanted traits

Induction of mutant gene will occur at random, but mutant character can be used as the screening element. For example, early flowering mutant can be found in M2 population rather easily. If appropriate screening method is established, resistant mutant plant(s) may also easily be found as a survivor in disease or pest inoculated screening field. This screening field should be developed before actual screening work. The survival screening may be very effective, but panicle to row planting may still be suggested.

As for a planting pattern, 1) a methodology developed by plant breeder, one grain, or a few grains, from one M1 panicle each may be practical, however, 2) if the researcher is on a starting stage of the mutant induction experiment, panicle to row planting is strongly suggested to have good experiences in the segregation in M2. After the visual experience on the Mendelian Segregation, researcher's may establish his/her own methodology. Theoretically, after mutagen treatment of multi-cellular seed embryo, the central stem may show chimerism. However, after full growth of a rice plant, chimerism might be much reduced. Among almost 20 or more panicles from a single treated seed, most of panicles show segregation ratio of one mutant to three normal plants.

For practical screening, the knowledge on where the expected mutant character will be expressed is very important.

1) If **endosperm character**, e.g. waxy starch, (also, sugary, floury, and giant embryo *etc*) is the target character, it will be expressed on the seeds produced on the M1 panicle. These seeds are in M2 generation (see files of PictureWord in this CD-ROM). The details may depend on the variety and the planting custom of the region, but in Japan, if a seed population is treated by a mutagen in spring, after harvesting and drying, screening or observation of the characters is possible in November or December of the same year. (Please note that this screening is made on the M2 seeds, so that the rule of M2 screening is still applied here.)

2) On the other hand, **plant structure** (semi-dwarf *etc*), **physiological characters** (early flowering *etc*) which are expressed in M2 plant or maternal tissue mutant like hair-less (glabrous) the target characters need to be examined in M2 field, where panicle family planting (at least 10 or more plants per panicle) is suggested. Segregation of more than two mutant plants in a panicle family gives good confidence to the observer. If the purpose is for academic studies where lethal mutant(s) are also important, then, to maintain the mutant gene as heterozygotes, this panicle family planting will be a necessity.

3) Some seed characters depend on the **plant structure**. A mutant character with twin pistils in each flower should be searched in the M2 plant population, or M3 seed population (twin grains may be seen as strange twin grains within a set of hulls). Some of **seed shape** of short round (in japonica) or a length mutant in indica lines, may be resulted from mutant characters of the housing of hulls. For these maternal tissue characters should be searched in the M2 plant grown on the 2nd year.

6) How to brush up the mutant

The number of genes in a species is said to be more than 10,000. Suppose the number of genes important in agriculture, as only 10,000, and mutagen treatment induces a gene at 0.1 % frequency, the one treatment may induce

10,000 x 0.1% (=1/1000) = 10

that is about ten mutant gene will be occurred simultaneously after one mutagen treatment. So, if you were successful in inducing a *sd*1 mutant or a good *wx* mutant, the promising mutant line may carry also nine other mutant genes induced simultaneously. Sometimes simultaneous mutant gene gives another very significant character like virus resistance in a barley early mutant in Japan or salt resistance in Milns Golden Promise (barley in UK). However, in most of the cases, they are albino or reduces seed fertility, that is unfavorable elements.

Rice plant has 12 chromosomes. So, it may be highly possible that the simultaneously induced genes may be dispersed on the different chromosomes. Actually one backcrossing to the original line easily excludes the unfavorable gene and seed fertility may be returned to normal level. Such backcrossing is not a cross breeding, but a necessary procedure before yield tests. It is only to retrieve the original genotype except the favorable new mutant gene.

Some Mutants for Hybrid Rice System

Induced mutations are recessive. So, it will not expressed in F1 hybrid generation. As far as the mutant is used in one side of the parent, any mutant may be used. Contrary, if one want to express a phenotype, both parent must carry the same gene, even if the mutant site (within the locus) may be different. For this purpose, mutagenesis will be a powerful means to have independent origin for the same gene/cistron.

Nuclear gene male sterility is relatively easy to get, but usually very difficult to apply to the Hybrid Rice system. Temperature dependent TGMS mutant in rice may be one good possibility (cf. Tripletcode.doc in Introduction).

Fertile twin grain mutant has two sets of pistils, suggesting better external pollen reception. This recessive mutation will not be expressed in the F1 hybrid generation, when ordinary seed shape is necessary.

Flower of the twin grain mutant has two pistils, both are fertile, if condition allows.

In rice the flowering time of the day is not long, a few hours in the morning. This open hull mutant keeps open its flowers, or can not close. However, this mutant has good seed fertility as shown in this picture.

As for the pollen parent, tall plant for better pollen dissipation is desired in *sd*1 background. For this special purpose, leaf color mutant as a marker gene was successfully used to induce "tall mutant but with *sd*1 background (cf. markergene.doc).

Finally, hybrid rice systems, three lines by cms and two lines by tgms or pgms(photoperiod dependent ms) may be illustrated as follows. For seed harvesting crops like rice, dominant *Rf* (fertility restorer) is a necessity in the three lines using cms, but such dominant gene can not be induced by mutagenesis. Tgms or pgms does not require *Rf*, but practical problems may need solutions.

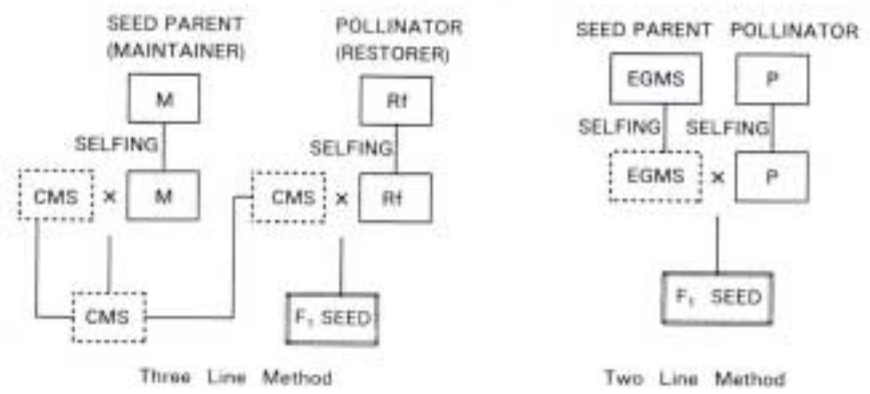


Fig. 1 Comparison of F_1 seed production system by CMS and EGMS
Three Line Method : F_1 seed production system by cytoplasmic male sterility (CMS) and restorer gene (Rf)
Two Line Method : F_1 seed production system by evironment-sensitive genic male sterility (EGMS)

Lodging has been a big problem in many crop plants. After heavy wind, near harvest time, highly valued "Koshihikari" often lodges.

The first Lodging resistant mutant variety was developed from 'Fujiminori'(left) and registered as 'Reimei'. The new variety was named in the hope of successful application of mutation in crop breeding, meaning 'the dawn' or 'day-breake'

Ten years later, a comparable American variety "Calrose-76" followed as the same lodging resistant variety developed by Dr. Rutger (Picture.) and his group. The semi-dwarf gene sd_1 was identified in "Reimei", "Calrose-76" and the famous "IR-8".
(Copied from IAEA publication)

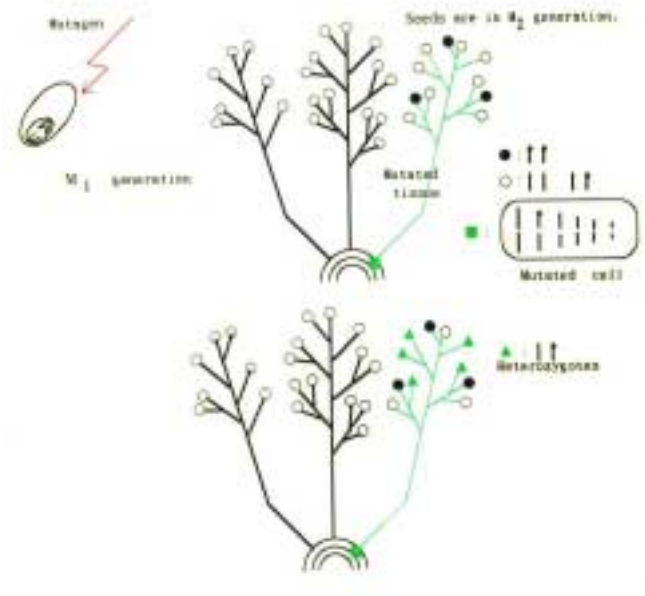
The use of Mutation Breeding in Japanese Rice.

After successful release of lodging resistant 'Reimei', so called direct use of mutant amounted to about 20 and indirect use (use of mutant line in cross breeding) amounted to almost 50 varieties. For examples, low amylose (sticky but not fully waxy) line 'Milky queen' is bred as direct use of mutant, and strong culm semi-dwarf 'Kinu-hikari', giant embryo cultivar 'Hai-minori', and low gluterin (good for allergic patients) 'LGC-1' are examples of use in cross breeding.

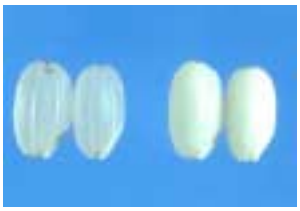
Recent low amylose rice cultivars in Japan

Mutant	Progeny	Mutagen
NM391		Gamma-ray induced
	'Asa-tuyu'	
	'Aya'	
	'Hanabusa'	
	'Ayahime'	
74wx2N1		EMS induced
	'Snow Pearl'	
	'Takitate'	
74wx8N1		EMS induced
	'Akane-Fuji'	
'Soft 158'		EMS induced
'Milky Queen'		NMU induced
'Yawara-Komachi'		NMU induced
'Silky Pearl'		NMU induced

Mutants screened in M_1 panicle.



When multi-cellular seed embryo is treated with mutagen (top left), the central axis panicle may have chimeric structures, but later coming panicles may segregate within the entire panicle. Please note that the seeds on the panicle are the following generation and the seeds are in M2 generation, so endosperm characters or seed embryo characters are segregated on the M1 panicle as shown in the top right green panicle. Lower figure suggest also the presence of some heterozygotes. In case of academic studies, where lethal mutant may have some importance, panicle family planting is important to keep the heterozygotes for maintenance of the lethal mutant gene. A few examples are shown here.



Typical example of endosperm mutation may be waxy starch mutant (white endosperm ('Miyuki-Mochi') at right induced from Wx ('Toyo-Nishiki'). Glutinousness or amylose deficiency can be confirmed by iodine staining (see Starchmutant in this rice file).

Lighting behind the seeds shows normal (left two grains) and giant embryo (right upper two grains) and turbid wx grains (lower two grains). Comparable mutant line of giant embryo has been registered as 'Hai-Minori' in Japan. Half way germinating seeds contains many excellent nourishing components, good for health. The character is relatively frequent after chemical mutagen treatments.

The giant embryo mutant has various nourishing components in the enlarged embryo. The mutant has registered as "Haiminori" in Japan (Left picture. Right picture is ordinary Koshihikari for comparison).

This picture may be comparable to sugary mutant in maize, the sweet corn, although use in rice has not developed yet.

Mutant character selectable in M2 generation.

Albino, abnormal dwarf etc. may be easily come to ones mind. Here other cases of important characters are illustrated as examples.

Early flowering mutants may also come to the mind. These are plant characters detectable in M2 generation. This picture shows two early flowering mutant lines segregated after callus culture mutagenesis, one in left-back and the other right front.

Some tissues are maternal tissues, so that the mutant phenotype of them can be screened only in M2 generation. This mutant keeps the flower open, or does not close at all, but still seeds are fertile as seen in the next picture.

Many "open hull" mutants are seed-sterile, but in this case, seeds are seen in the picture. It may be a promising mutant for hybrid-rice system for better pollen reception. It is recessive and will not expressed in F1.

Another case of promising mutant for hybrid rice system may be this "twin pistil" mutant. At first, strange shape of seeds were noticed. The shape is not good for ordinary grain polishing. But the detailed observation showed that the mutant plants have two sets of pistils, both seemed to be fertile if condition allows.

The male organs (anthers) are normal and the female organ (pistil) is basically fertile to give twin seeds. It suggests that the pollen reception may be twice as much as normal single pistil line. Seed fertility of more than 130 % had been nominally recorded on the fertile seed embryo per flower. This character is recessive and will not be expressed in the F1 generation, suggesting this may be a good character as the maternal line in the hybrid rice system. These characters expressed in the maternal tissues may be screened in the M2 generation field (in case of the "twin grains" flowers in M2 plants, and seeds shown above are in M3 generation).

Sometimes, purely academic research demands special mutant line which may not be useful in agriculture. For such academic purposes, mutants are very often requested from researchers. This picture is a rice panicle mutant, the panicle branches seemed to continue branching resulting in no flowers at all on the panicle. It has been induced by gamma-rays and also by EMS. The sterile mutant can be maintained as heterozygotes.

This picture shows two types of the abnormal panicle mutants. The right one is the same as shown above, but the left one was induced after callus culture.

Use of viable and fertile mutant as marker gene.

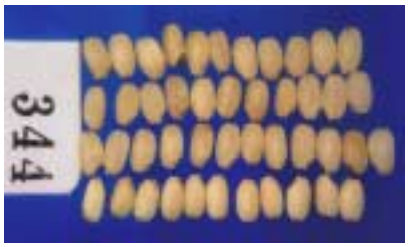
Rice is basically a self pollinating plant, however, the mutational event may be much lower than the assured self pollinating rate. Therefore in the M1 planting field like this picture (upper left light green(variety 'Reimei') and lower right light green(variety 'Norin-8')) previously induced and increased light green and fertile "*chlorina*" mutants were used to assure the line purity.

Even surrounded by other normal varieties, the gene-marked lines may give the clear characteristics of the line, in this case, light green color. Contaminated plant with normal green color may be taken out from the seedling bed. In the right side, purple seedlings are seen, but these are dominant purple line in which contaminated normal green plants may not appear or hidden by the dominant purple gene.

When you can get a promising mutant, this recessive *chlorina* marker gene may be excluded easily from the promising mutant lines, by a single back-cross to the original line.

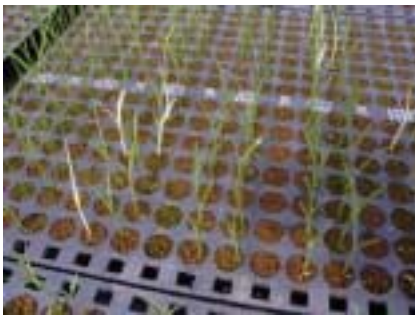
After transplanting to the main field, still floating seedling may come from neighbor group. Such normal green plant may be excluded from the selection field. By such very strict field maintenance, a tall mutant (recessive to sd_1) with sd_1 ('Reimei') background was selected.

Some examples of Rice Mutation Experiments.



Segregation of waxy starch grains (the lowest line) in a japonica variety. Generally, later panicles segregate 3 normal and 1 mutant grains. In japonica variety, some heterozygotes look half-waxy, e.g. 2nd and 3rd grains in the top row. The next fourth grain has a defect embryo.

The left two grains are the original normal grains. Upper right two grains are giant embryo (perhaps same type as "Hai-Minori" variety). The lower two grains are waxy starch mutant. This picture was taken by back-illumination.



Albino seedlings indicate that the mutagenic treatment had been effective. Fourteen holes were used for one M1 panicle family, skipping a row of the next seven holes as separation between families.

An example of M2 field planted with panicle to row system. Indicator poles show some kind of mutants were segregated in the family line.

Two early flowering mutants are shown here as they segregated in panicle to row planting in a M2 field.

Two or three dwarf mutants are segregated as indicated by the pole.

This is a case of disease susceptible mutants shown by the pole. In a practical breeding, this may be selected out, but for academic research work, this may be important material to study the infection system of the disease.

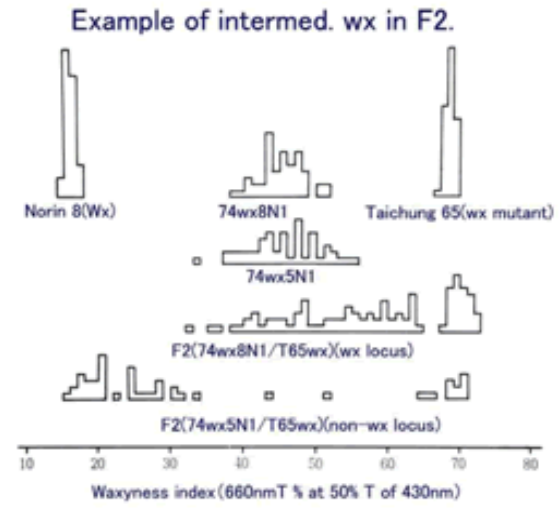
When harvesting in a panicle to row field, normally looking (possible-)heterozygotes are important material if academic research work is intended.

Starch mutant in rice

In rice, seeds produced on M1plant can be used for starch type mutation screening. The result shown here may look continuous variation from non-waxy (left) to complete waxy (right). Extra grains in top row indicate Controls.

Starch can be stained by iodine solution, normal non waxy blue and waxy mutant

Starch components can be easily separated by a simple paper chromatography run by 1M KOH. The normal non-waxy starch has amylose, the bluish spot in the upper part, but waxy mutant (Right lane) lacks it.



Intermediate phenotypes were further studied and genetic analyses made clear that 74wx8N1 was a *wx* locus mutant, but 74wx5N1 was low amylose mutant but other than the *wx* locus.

2. Barley and wheat

Barley is diploid and ordinary (bread) wheat is hexaploid. In a hexaploid crops, albino may not show up in M2 generation by the function of triplicated homeologous genes in the three genoms (A, B, and D). Except such difficulties, handling of the seeds may be quite the same way in the two cereals. So, here, barley may be the example.
In wheat, ionizing irradiation often induces chromosome aberrations. Unless such aberration is the target of the experiments, watch the seed fertility, especially after backcrossing to the original line. Translocation line may continue lower seed fertility in the BF1, but if the line was aberration free, seed fertility may return to normal.
Basically the method for rice may be followed, but number of seeds per ear/spike, and the number of tillers per M1 plant, will be much smaller than those of rice plant.

1) How to prepare starting materials

The breeding method using induced mutant(s) will improve only a few characters, e.g. semi-dwarf (for lodging resistance) or early flowering or waxy starch, leaving other characters unchanged at all. So, this method may be applied best to a local varieties. Many kinds of mutant characters will be found during screening procedures. Therefore, it may be necessary to have a clear objective of the expected improvement. Otherwise, many newly induced characters may draw attention of the observer, finally giving too much work on unnecessary characters.
To prepare the starting population (materials), if possible, isolation by paper bagging *etc* may be strongly recommended.
Use of hybrid seeds in mutagenesis is not a good idea, unless specific purpose of chromosomal translocation is intended.

> "Hybridization enlarges the possibility of mutation by enlarged number of target gene" is erroneous, as almost all the mutant gene is already inactivated so that further mutation will not give mutant gene at all. Furthermore, hybrid of mutant gene wx-a and mutant gene wx-b may produce completely normal Wx gene by intra-cistronic recombination even without mutagen treatment, at the rate of one in 10,000 or so, depending on the distance between wx-a and wx-b. Such result may be mistaken as a dominant mutation.

Keep some seeds for future backcross.
Use some seeds for treatment control to examine the treatment dose and other procedures. This may be important when you send out seeds to other institution for treatment/irradiation. A small control seeds to examine the transportation condition is important sometime.
Many of barley and wheat varieties may need some kind of cold treatments (vernalization) to give flower buds. The need of such treatment is needed in M1 and M2 generations (unless vernalization free mutant is sought in M2 generation (even if such was the case, M1 must be still in the favorable condition).

2) Mutagens suggested

This depends on the purpose of the work.
If any kind of mutation is accepted for crop improvement program, irradiation by X-rays or gamma-rays, or if you have a small chemical laboratory, chemical mutagen e.g. EMS may give a good result. In case of Sodium Azide[1], please consult to appropriate literature for necessary pH and/or growth stage to apply.
If **Deletion mutation** is wanted, intended to extract a specific mutant gene, ion beam, reactor neutrons or other appropriate high-LET radiation is suggested. In some radiation, e.g. fast and/or thermal neutrons, small but appreciable amount of radioactivity will be induced by irradiation. Special care should be taken in practical handling and also to meet regal restrictions.
If **intermediate or leaky type mutation** is wanted, chemical mutagen, e.g. EMS, which may cause base-pair substitution mutation may be suggested.
The idea will be extended to other special type mutation, like **temperature dependent mutations**, which may be explained by temperature dependent 3-dimentional folding of protein molecules, the gene product, although it is not proved yet. If the explanation proved to be true, chemical mutagens to induce base-pair substitution may yield such mutation, perhaps.

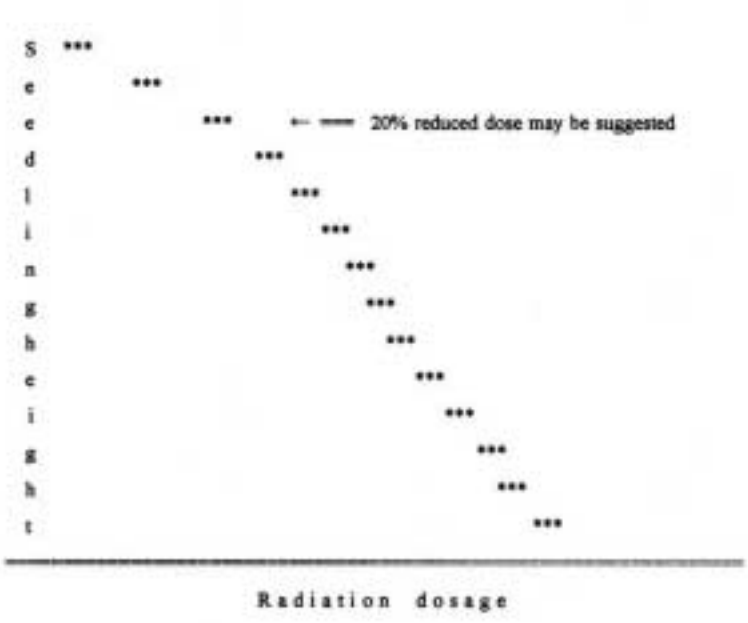
3) How to decide treatment dose

This may be the very important procedure in the mutagenesis experiment. The sensitivity tests are different for radiations and chemical mutagen which is usually applied as water solution.

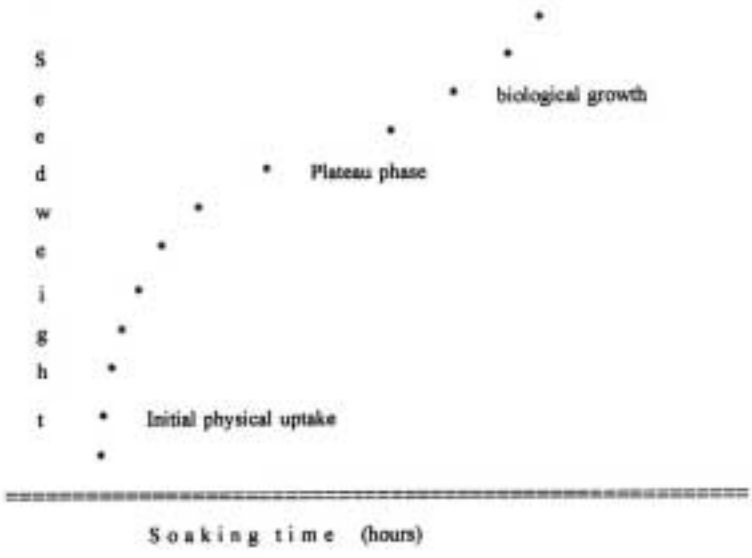
Irradiation

In case of irradiation treatment, sensitivity differentiation is reported in some materials perhaps by a few sensitivity genes. In barley, the two types of "hulled" and "naked" type seems also different in the radio-sensitivity, perhaps due to radio-sensitivity gene(s).

To decide appropriate dose, seedling height measurements from 100 Gy to 700 Gy, to cover almost no effect to heavily damaging dose should be tested at first, in 7 or more dose classes with up to 20 seeds each (see the same in "1rice"). About 20 % reduced dose may be suggested as recommendable dose, where M1 seed set is not much reduced. For radio-sensitivity comparisons, LD-50 (50 %lethal dose) or RD-50 (50 %Reduction dose e.g. by fresh weight) are often used as critical values to compare. However, for mutation induction, such doses are too high for better seed fertility and especially for less accompanied mutation.



For chemical mutagens, another curve to show the water imbibitions is important to decide the treatment.



Dry seeds show very fast water uptake in the initial several minutes, then reach to a plateau, where chemical treatment (soaking) is suggested. The strength (concentration) test of the water solution may be comparable to the radiation test shown above. Please be aware of the temperature. In barley and wheat, 15 to 20 ℃ presoaking and soaking treatment may be suggested.

Ethyl methanesulfonate (EMS) treatment may be very effective. One example of a recipe may be as follows :

Presoaking in distilled water at 15℃ for two hours.

Soaking treatment in 1% EMS at 15℃ for 5 hours while shaking gently.

Post wash a few times then keep in dist. water over night.

After final washing, seeds may be sown to a seedling bed.

All paper and glass wares and rinsing water used in the treatment should be put into a bucket of appropriate NaOH solution (around 0.1 %) to hydrolyze the carcinogenic mutagen.

4) How to grow and harvest the M1 generation

After the mutagen treatment, barley or wheat seeds of M1 generation may be planted in field according to the local procedure. Genetically speaking, each treated seeds should be a separate unit in terms of mutagenesis, so one seedling per hill/hole may be suggested. If one plant per hill can not be attained, use much more seeds for mutagen treatment and harvest one spike per hill, neglecting other seeds in the same hill. The purpose of planting M1 generation is to advance the generation to M2 by self pollination. So, plants should be maintained to obtain good seeds using various pest controlling procedures. Do not attempt to put any kind of screening yet in the M1, because the induced mutant gene (recessive) is not fully expressed at this stage. Occasionally chlorophyll mutant stripe may be observed in few plants among thousands of M1 (diploid) plants. This may be a simultaneous two mutations at the same chlorophyll gene (paternal and maternal) locus. Most of it will be expressed only on some leaves, but it may be a good indication of successful mutagenesis treatment.

Suppose a mutation frequency of a gene as 0.1 % or one in 1000, a possibility of two mutations in the paternal and maternal genes at the same time will be 0.1 % x 0.1 % = one in 1,000,000. Leaf primodial cells in a seed embryo may be amount to 1000 or more. Thus this may be visualized in 1,000,000 treated cell population or a few stripes in thousands of plants.

The central panicle may have chimerism, but most of later panicles seem to be practically free from chimerism. Supposing the panicle-wise screening in M2 generation, suggested harvesting system is as follows, (the same as suggested in rice) each group should carry name tags accordingly :

1) **The First Round Harvest :** Harvest only one panicle per hill, all through the M1 population. In all the harvested materials, don't forget to place name tags in the way available in the local condition. This harvest may be used to examine endosperm characters. It may be difficult to keep the seeds separately after seed character examination, normally looking seeds may be discarded.

In Washington State Univ. (Pullman), Prof. Nilans group was once successful to get several waxy barley endosperm by iodine staining in the half grain system, where embryonic half is kept for germination.

2) **The Second Round Harvest :** In the same way, second round harvest should be made again from the beginning of the M1 population. Some of the panicles may genetically duplicate from the First Round Harvest, but if you use this in a different category like plant type characters expressed after germination, duplication is not a problem.

3) **The Third Round Harvest :** Because M1 generation planting is laborious work, this 3rd round harvest may be suggested for storing for future work. Store the spikes in dry and cool condition for future use. If seeds are easily dropping off from spikes, many paper bags may be needed to store the spike families separately.

5) How to screen the wanted traits

Induction of mutant gene will occur at random, but mutant character can be used as the screening element. For example, early heading/flowering mutant can be found in M2 population rather easily. If appropriate screening method is established, resistant mutant plant(s) may also easily be found as a survivor in disease or pest inoculated screening field. This screening field should be developed before actual screening work. This survival screening may be very effective, but still panicle to row planting, description of which is just following, may still be suggested.

As for a planting pattern, 1) a methodology developed by plant breeder, one grain, or a few grains, from one M1 panicle each have been proposed, 2) however, if the researcher is on a starting stage of the mutant induction experiment, panicle to row planting is strongly recommended. After the visual experience on the Mendelian Segregation, researcher's own methodology may be developed. Theoretically, after mutagen treatment of multi-cellular seed embryo, the central stem may show chimerism. However, after full growth of the plant, chimerism might be much reduced, among almost five or more spikes from a single treated seed, most of panicles may show segregation ratio of one mutant to three normal plants.

For practical screening, the knowledge on which tissue the expected mutant character will be expressed is very important.

1) If **endosperm character**, e.g. waxy starch, (also, sugary, floury etc) is the target character, it will be expressed on the seeds produced on the M1 spike. These seeds are in M2 generation. The details may depend on the variety and the planting custom of the region, but in Japan, if a seed population is treated by a mutagen in autumn, after harvesting and drying, screening or observation of the characters is possible in May or June of the following year.

2) On the other hand, **plant structure** (semi-dwarf etc) or **physiological characters** (early flowering, no-need of vernalization etc) which are expressed in M2 plant, the target characters need to be observed in M2 field, where spike family planting (at least 10 or more plants per spike) is suggested. Segregation of more than two mutant plants in a spike family gives good confidence to the observer. If the purpose is for academic studies where lethal mutant(s) are also important, then, to maintain the mutant as heterozygotes, this panicle family planting will be a necessity.

6) How to brush up the mutant

The number of genes in a species is said to be more than 10,000. Suppose the number of genes important in agriculture, as only 10,000, and mutagen treatment induces a gene at 0.1 % frequency, the one treatment may induce

$$10{,}000 \times 0.1\,\% \,(=1/1000) = 10$$

that is about ten mutant gene will occur after one mutagen treatment. So, if you were successful in inducing a semi-dwarf mutant or a good quality mutant, the promising mutant gene may carry also nine other mutant genes induced simultaneously. Sometimes simultaneous mutant gene gives another very significant character, e.g. virus resistance in a barley early mutant in Japan, or salt resistance, in Milns Golden Promise, in UK. However, in most of the cases, they may be albino or reduces seed fertility, which is unfavorable element.

Barley plant has seven chromosomes, and wheat has 21 in haploid states. So, it may be highly possible that the simultaneously induced genes may be dispersed on the different chromosomes. Actually one backcrossing to the original line easily excludes the unfavorable gene and seed fertility may be returned to normal level.

Such backcrossing is not a cross breeding. It is only to retrieve the original genotype except the favorable new mutant gene.

As previously mentioned, chromosome aberration might occur in wheat. If BF1 is still shown low seed fertility, it may have chromosome aberration.

Mutant segregation and screening work

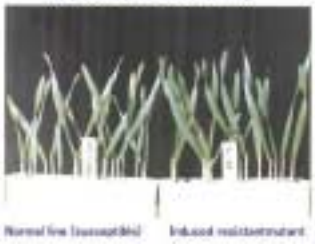
Bread wheat is hexaploid (6X), but for academic studies diploid species are used like here at the National Institute of Genetics, Misima in 1970s. In hexaploid species, albino seedling is difficult to induce and to show, but diploid species shows albino seedlings.
The spikes (or ears) harvested from M1 plants are put in the soil, so that spike family will show up together like the following two pictures.

The second picture is the case of light green seedling mutants segregated

.
The albino, also shows red pigment still it contains.

Barley is a standard study plant for mutagenesis. This picture was taken in 1960s at Brookhaven National Laboratory in the USA (by E.Amano). The barley spikes harvested from M1 (seedlings are in M2) were put into the sand bed like the case of the diploid wheat shown previously. The mutagen used by Dr. R. Heiner (previously Washington State Univ.) was dES.

M2 seeding bed for screening



After many preliminary tests, practical disease resistant mutant may be screened like the one done at the Institute of Radiation Breeding, Ohmiya-machi, Japan.. About 120 million M2 seedlings were germinated and used in screening of "mildew" resistant mutant and several resistant lines could be selected by this screening.

This mutant line shows disease resistance against the soil born BYMV (barley yellow mosaic virus). The surrounding lines were clearly damaged by the virus disease. This resistant mutant was at first selected as early maturing mutant, but later it was found to be a disease resistant line to the virus as seen here by good growth. This might be a favorable case of simultaneously induced mutation. The mutant was registered later as 'Masakado-mugi' in Japan.

3. Maize

Maize is basically out crossing crop having male flower tassel at the top of a stem and female cob(s) are in the lower stem node(s). The seed embryos are already multi-cellular in its structure, so that the seed treatment gives chimerism, separating the male tassel and the female cob. Therefore special strategy should be set in mutagenesis and screening.

Another very strange characteristics of this crop plant may be the fact that ionizing radiations usually does not give good mutant. It induces deletion mutations of various sizes, generally large and unfavorable size for agricultural performances (Stadler and Roman 1948, Mottinger 1970 and Amano 1968, 1972). The chimerism in the M_1 plant and very strange reaction to the ionizing radiation make this important crop a very difficult material for mutation work. However, if the chimerism pattern is understood and appropriate mutagen (chemical mutagen, EMS, may be suggested) could be used, mutation work in this crop may give good results, both in academic work and agronomical applications. In the IAEA mutant cultivar data bases, on Internet system and the attached database based on the IAEA publications, many mutant cultivars were registered, as the system accepted the reported content without confirmation. However, perhaps only chemical mutagen induced data might be dependable (refer to Mottinger 1970 Genetics 64:259-271, or Amano 1985 Gamma-Field Symposia 24:81-96).

The basic handling of this plant in pollination system should be followed in the mutation work.

1) Removal of male tassels may be depend on the project purpose, but detasseling and pollination by use of a paint brush may be helpful in mass pollination work.
   a) If marker gene system can be used, remove male tassels (detasseling) for better pollination work.
   For example, if new waxy starch mutant is wanted in an inbred line, mix waxy line pollen with the original inbred line in equal amount (e.g. by weight) and place pollen onto the isolated silk with dry painting brush. Mutated waxy female cob will show 1 waxy and 3 non-waxy seeds. The non-waxy seeds may be composed of 1) +/wx-line, 2) +/+(original) and 3) wx-induced (with original background)/+(the original). The last one will give inbred waxy mutant line by self pollination in the next generation.
   It may sound complicated, but this kind of strategy is very effective if it should be well planned beforehand.
   b) If a new character is sought, leave male tassel and perhaps free open pollination may be used in M_1, if isolated plot can be used for the M1 generation.
   Observation of chlorophyll sectoring in the male tassels which suggests that each chimeric unit might be as large as a single brunch of a male tassel. So, about 10 or more mutant sectors in a tassel.
   In a seed-EMS treatment experiments, female cobs, most of the mutated seeds, e.g. colored (from C-I to C) seeds distributed rather evenly on the cob. However, there was one case, where only one clear mutant seed was found at the bottom of a cob. This may mean that the small mutated sector was only at the base of the cob and one grain was shown as 1 mutant against 3 normal seeds. Therefore, circular sampling at the base of a cob may contain more mutant sectors than a row sampling from bottom to top.

1) How to prepare starting materials

In maize, commercial seeds are either hybrid seeds or inbred. Hybrid seeds will give good performance in commercial cultivation, but for plant breeding work, only inbred line may be meaningful. For mutation work, perhaps improvement of local (inbred) varieties may be a good target.

Well controlled hand pollination at least one generation and uniform appearance of seeds on any cob, should be confirmed. The improved line then may be used as parental line for "hybrid seed system".

The idea of "Hybrid seeds enlarge the target gene number" is erroneous. Mutant genes are mostly inactivated, or in other words they are already broken, so the effective target genes of a hybrid is reduced than pure inbred line. AA genotype has two targets, A and A, but Aa has only one target A.

Isolated experimental plot may be needed for mutation work. Please be noted that maize pollen grains may fly away as far as hundreds of meters.

2) Mutagens suggested

As described before, maize reacts to physical mutagens in a strange way. Maize can repair the chromosomal damages (e.g. chromosomal breakage) as other plant species, but the timing of the repair seems to be different from other plants. This phenomenon has been reported by L. J. Stadler (1948 Genetics 33:273-303 etc.), J. P. Mottinger (1970 Genetics 64: 259-271) and E. Amano (2001 Gammer Field Symposia No. 40 35-47). Personally, during my more than 20 years of experiences, no good viable mutants could be available in maize by gamma-, X-, fast-neutrons and thermal neutron irradiations. Germicidal UV to pollen grains was successful but details are still left to be studied.

Compared to these radiations, chemical mutagen tested, e.g. EMS was very successful in Wx, Sh, C-I loci to give various good and viable recessive mutant lines. In these induced mutants, intermediate phenotype (mis-sense or leaky type) mutations were often observed, but in most of the cases they were clear "complete" type mutants.

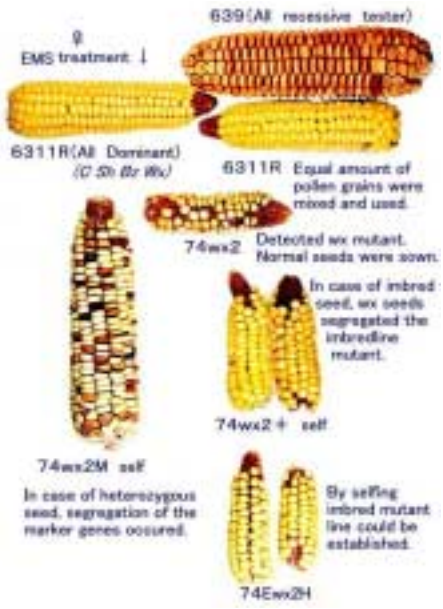
3) How to decide treatment dose

The methodologies and evaluation of the results are same as those for rice or barley. So, refer to those methods, shown elsewhere. But please be noted that the dose may differ from rice, or often among the maize lines. So, if you are starting the mutagenesis work, it will be better to examine the dose-response curve by yourself on your material. Ten to 20 seeds per dose class may be good enough, but the number of dose classes being five to ten, extending from 10Gy to significant damaging, e.g. 400Gy or so.

4) How to grow and harvest the M1 generation

Isolation plot may be used, or if it is not available, isolation by bagging should be done. However, other handling in the M_1 field may be planned only to obtain M_2 seeds. Depending on the purpose and strategies, open pollination may be accepted if an isolated plot is available.

4-1) Use of genetic marker : If marker-use-pollination (e.g. searching of waxy mutant) is used to induce the same mutant character in an inbred line, the mutant character may be examined on the cob of M_1 plant or on M_2 seedling/plant. Perhaps you are treating inbred dominant line. Prepare both the tester line (e.g. inbred wx/wx line) and the inbred original line. Collect pollen from both tester and the inbred parental line. Mix equal amount and put on the silk of the treated female. By this way, you can detect the intended mutant kernels on the female cob. On the cob, normal looking kernels are either non-mutant kernel (+/tester wx) or inbred kernels (wx/+ or +/+). In the following generation, by self pllonation within the plant these normal looking kernels may give segregation of wx or +/+. If the plant give good growth by heterosis, it might be hybrid seeds with the tester. If the same growth as the inbred parent and segregating the intended mutant gene, you are successful in obtaining the inbred-line-mutant.



4-2) Unless the marker-use-pollination is utilized, mutant screening may be done in the M_3 generation after self pollination of M_2 plants. The chimeric mutant sector might be most possibly locate in the base of the cobs. So, plant the M2 seeds collected from the base of the cobs, two rows around the cobs. The total population will amount to about 20 grains times M1 cobs. Each M2 plants must be self pollinated within the plant. If this was the method you are adopting, then the M1 plants may be open pollinated, provided if you get good isolation environment.

5) How to screen the wanted traits

If marker-use-pollination is used to induce the same mutant character in an inbred line, the mutant character may be examined on the cob of M1 plant (in cases of endosperm chracters) or on M_2 seedling/plant.

Unless the marker-use-pollination is utilized, mutant screening may be done in the M_3 generation after self pollination of M_2 plants. As described before, circular sampling at the bottom of M_1 cob may be recommended to grow M_2 plants, which should be self pollinated to give M_3 seeds/plants for screening. Screening techniques may differ from character to character, e.g. disease resistance needs active inoculation or disease affected environment.

6) How to brush up the mutant

Simultaneous mutation might occur in the treated materials. Therefore, at least one back cross should be tried to exclude unfavorable additional mutation. As far as the original parental line is used, general genetic background is maintained as the inbred line. This "back-crossing" is not disturbing the genetic background much, so it should be separated from cross breeding. The use of the mutant line may be still the direct use of the mutant (not indirect use).

Mutagenesis in maize (Chimerism)

Mutagenesis in maize needs some special considerations. There may be two important problems regarding maize mutagenesis 1) appropriate mutagen and 2) chimerism. For mutagens suggested, see the other file "**Mutagenmaize".**

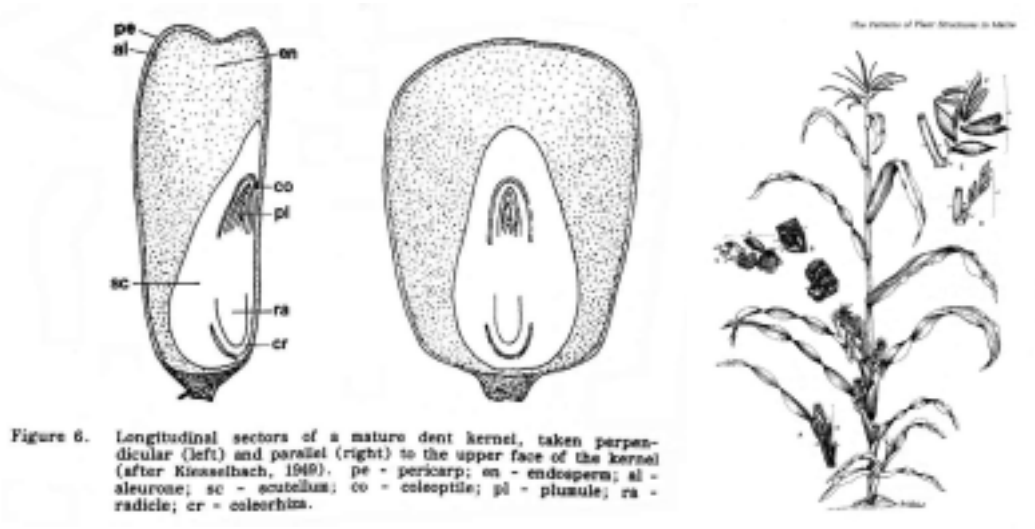


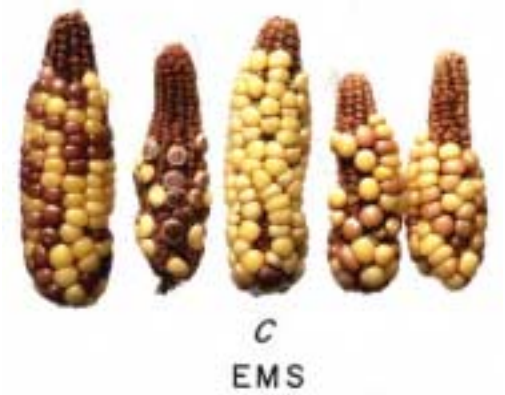
Figure 6. Longitudinal sectors of a mature dent kernel, taken perpendicular (left) and parallel (right) to the upper face of the kernel (after Kiesselbach, 1949). pe - pericarp; en - endosperm; al - aleurone; sc - scutellum; co - coleoptile; pl - plumule; ra - radicle; cr - coleorhiza.

In cases of rice or barley, self pollination of the M1 plant may be mostly real self pollination within the flower. However, in maize, when the seed was treated by mutagen, the embryo is already in multi-cellular structure. The mutant tissues may have developed already, the top male tassel primodial cells had been separated from the lower node female flower primodial cells, making <u>self pollination within the plant as not-real-self-pollination</u> in the sense of mutagenesis. This is shown in the color pictures in this file.
Seed embryo had been developed in multi-cellular structure.

This picture shows the presence of mutant tissue development by the leaf color. When a pure line was treated, generally no such mutant tissues develops in the M1. But sometimes at a rate of a few cells in the millions of mutational unit (cells) double mutation of the same gene locus might occur to give mutant sector like this. The frequency of such sectors are very low, but it suggest successful treatment, as it occurred in rice or tomato, too.

In this picture, pure white sectors developed on a tiller or branch, meanwhile the plant shows light green sectors on one of the male tassel branch. These mutation might be independent events as seen in the color difference.

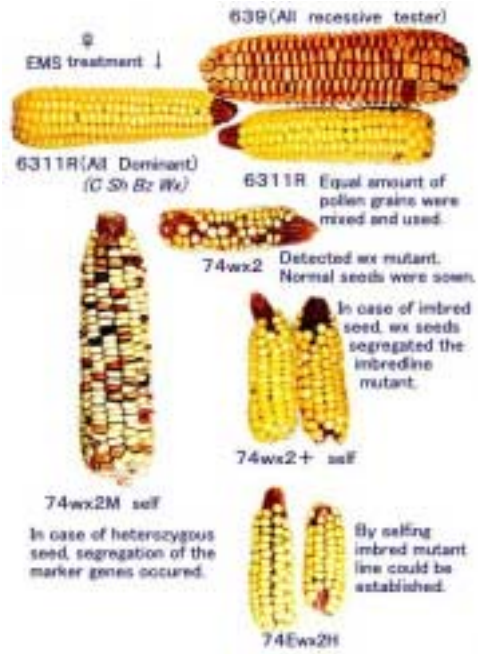
Here the mutant sectors on the male tassel is albino or white.



This is the detected *C* mutation from *C-I, color inhibitor* gene. All dominant (*C-I*, *Sh, Bz, Wx*) was treated and pollinated with all recessive tester. This picture shows the cases of *C* (color) mutations. Please note the cob at the center has one purple colored kernel at the base of the cob, suggesting that the mutant sector came in only here, and the rest left un-mutated. The two cobs at right might be the cases of intermediate (leaky or mis-sense) mutation giving lighter but clearly light purple color.

Waxy gene is a precious marker gene in genetics and mutagenesis work, as it is expressed both in the endosperm and in pollen grains. Further more the waxy starch has been sought in the East Asian people, in rice, sorghum, millet and other cereals. The waxy gene used in the maize genetics are said to be found in the collection from North-East region of China. Here in this picture, the treatment and the detection was done as the case of the *C* mutation, and segregating 1 to1, as explained above.

Thus detected mutant grains are the hybrid between the induced *wx* and the tester *wx*. This "heterozygous status make the isolation/extraction of the newly induced gene very difficult. Therefore special pollination methodology was developed to isolate the "imbred line mutant" by use of the marker gene.

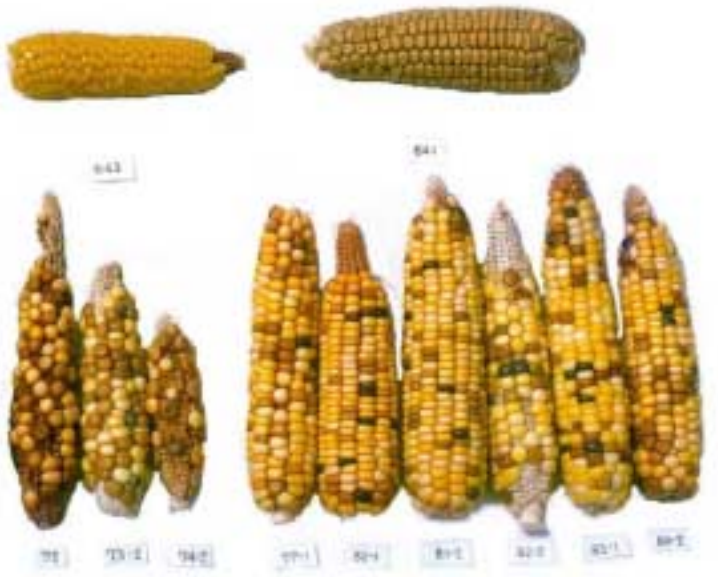


The mutagenesis treatment to the all dominant line and pollination with a tester were basically the same. But the pollen grains used to detect the mutant was an equal mixture of pollen grains of the tester and non-treated imbred line. The tester pollen, 639, and the pollen from original non-treated 6311R were measured, using a small balance, equal weight, mixed and used to pollinate using a dry painting brush. The detected mutant cob produced 3:1 segregation, in stead of 1:1 as shown previously. Among the normal segregants, some were heterozygote with the tester, but others were inbred line (sib pollination) and some segregated the induced wx seeds which will be further isolated as an "inbred line mutant".

Problems here may be the use of specific marker gene. In case of such marker gene is not available like a case of new disease resistance, this system may not be applied. Please refer to the description in the text part.

Mutagenesis in maize (mutagen)

Mutagenesis in maize needs some special considerations. There may be two important problems regarding maize mutagenesis, 1) appropriate mutagen and 2) chimerism. For Chimerism problems, see the other file "**Maize Chimera etc."**

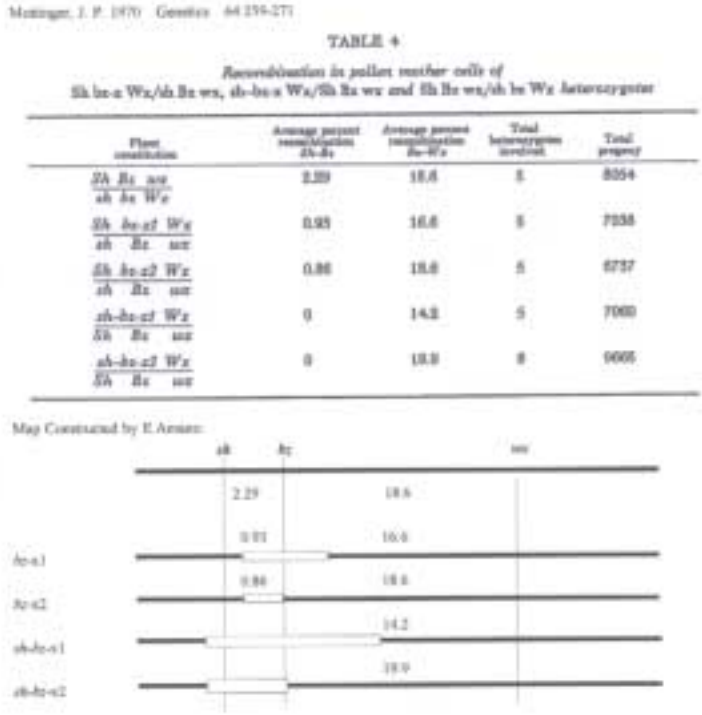


This list shows some personal results of E. Amano. In maize, ionizing radiations had produced several detectable mutations, but no mutant line survived in later experiments as shown in the cob pictures shown here. After mutagen treatment of top left material, *C-I, Sh, Bz, Wx* (all dominant), the M1 plant was pollinated by the top right tester (*C, sh, bz, wx*). Detected *wx* mutants were planted and self pollinated. Lower left three cobs are fast neutron induced *wx* and right six cobs are EMS induced *wx* mutants. The segregation pattern and seed sets are quite normal in the right group, but the radiation induced three cobs show less seed fertility and modifyed segregation, suggesting retarded survival of the radiation induced *wx* gene/chromosome (Amano *et al*. 1965Mut.Res., Amano 1972 GFSymp).

Mutagen	Years	Cobs	Mutation					wx freq.
			C	sh_1	wx	mult.	y etc.	%
X-, γ-	8	3929	6	3	1	1	2	0.025
N_f	4	1985	6	5	5	2	9	0.101
N_{th}	3	453	0	0	0	0		0.0
UV	7	(2253)	0	(7)	2	0		(0.089)
EMS	12	8188	48	56	78	1	15	0.953
NG	1	155	0	0	0			0.0

(Up to 1975 harvest)

This strange reaction of maize plant had been pointed out around 1950 by the pioneer researcher L. J. Stadler. Compared to fails to get good mutant by radiations, chemical mutagens, EMS, was very effective producing even intermediate phenotypes (leaky or mis-sense mutation). Nitroso-guanidine is said to be a highly effective mutagen in other organisms like bacteria, did not show promising results although it was only a preliminary trial.



Mottinger, J. P. 1970 Genetics 64:259-271

TABLE 4

Recombination in pollen mother cells of
Sh bz-a Wx/sh Bz wx, sh-bz-a Wx/Sh Bz wx and Sh Bz wx/sh bz Wx heterozygotes

Plant constitution	Average percent recombination Sh-Bz	Average percent recombination Bz-Wx	Total heterozygotes involved	Total progeny
$\frac{Sh\ Bz\ wx}{sh\ bz\ Wx}$	2.29	18.6	5	8054
$\frac{Sh\ bz\text{-}a1\ Wx}{sh\ Bz\ wx}$	0.93	16.6	5	7038
$\frac{Sh\ bz\text{-}a2\ Wx}{sh\ Bz\ wx}$	0.86	18.8	5	6737
$\frac{sh\text{-}bz\text{-}a1\ Wx}{Sh\ Bz\ wx}$	0	14.2	5	7000
$\frac{sh\text{-}bz\text{-}a2\ Wx}{Sh\ Bz\ wx}$	0	18.9	8	9005

Map Constructed by E.Amano

Further detailed report was referred here the one by J.P. Mottinger in 1970 (in Genetics). The lower part illustration is made (by E.AMANO) according to the list shown in the report (upper part). The report suggested that out of several analyzable mutant lines showed that they were still some size of deletion mutants.

4. Tomato (also *Capsicum* peppers etc)

Tomato and *Capsicum* peppers may look very different from rice, but genetically it is diploid, self-compatible and propagated by seeds, i.e. comparable to rice. Insects may mediate the pollen but basically they are self compatible plant. The difference from rice may be the objectives of breeding. The target of production of rice is the seeds, and fruits for tomato. In terms of mutagenesis and mutant selection, fundamental part for tomato and hot-pepper (*Capsicum* pepper) may be common with rice, e.g. reduction of growth by mutagen treatment may be comparable, except in rice, seedling height (i.e. length) is a good indication for treatment damage, but in tomato, fresh weight or dry weight (i.e. weight) increase after certain growth period should be evaluated.

In Japan, a variety of tomato was registered and released as "*In vitro* propagated variety". This variety `Koshi-no-Rubi` is "a half-way variety from cross to fixation", so that the seeds still segregate between the two parents, ordinary tomato and so called mini-tomato. This high quality fruit can be sold in big cities, but the seeds of it will show further segregation and can not reproduce the same high quality. Thus the major production area can continue to produce the high quality harvest as a local specialty, if *in vitro* produced young plants are restricted to the region.

Unfortunately, ordinary mutant variety has no such restriction mechanism. But the idea of limitation to certain region may be a good argument topic.

1) How to prepare starting materials

Some nurseries/seed-companies sell F_1 hybrid seeds for practical cultivation. They show very good agronomical performance by its heterosis as a hybrid (F_1). For mutation experiments, these hybrid seeds should not be used. Two independent recessive mutants of a specific locus may produce normal (dominant) recombinant seed at a rate comparable to spontaneous mutation frequency. Such recombinant normal seed may be easily misunderstood as "Dominant Mutation". Crop breeding by use of mutagenesis has its power in modification of only a few genes in the original genotype. Use of homozygous pure line is strongly advised.

Ideally, like in rice or in barley, the expected material line should be planted in isolation plot or isolation harvest by bagging isolation. Handling of harvested isolation seeds may follow the usual laboratory system. A dropped seeds should not be picked up, because such performance is the source of contamination in experiments in genetics. Breeding is a practical work and if good result is obtained regardless of the sources, contaminated seeds or well maintained, it is considered as a success. However, to keep high reproducibility, contaminated seeds should be carefully separated from the materials for mutagenesis.

If you continue the mutagenesis work for several years, a yellow green viable mutant detected in earlier experiment may be a very good genetically MARKED material to avoid possible contaminant seedling. If you are using homozygous yellow green mutant line and don't have any other yellow green line around, the contaminated seed/seedling will show normal green color at the seedling stage. The you may exclude from your experiments as contaminant. This method had been successfully used in rice (at Inst. Rad. Breed./Japan). Such marker gene may be easily excluded from the promising mutant line by back crossing.

Condition of the seeds, i.e., whether they had been kept in a desiccator of specific condition or not, may be important, if reproducibility of the experiment is important like the case of a pilot experiment of a project. However, if you simply want to have a favorable mutant line in a specific variety, such condition may not be important, if compared to the purity of the mutagenized materials.

In case of hot pepper, seeds should be carefully handled, perhaps by use of tweezers to avoid contamination of fingers by the very hot component of the fruits.

2) Mutagens suggested

The materials represented by tomato, dry and dormant seeds may be sent out to irradiation facility all over the world, if so wanted. In another words gamma-ray may be the most convenient mutagen for the seeds of this group. The flat seeds may also fit for the ion-beam irradiation where penetration of ion beams is often a restriction factor. As described in rice, in case of gamma-ray or X-ray irradiation, the secondary electron

equilibrium should be considered. Usually three mm thick plastic plate (acryl-plate *etc*.) covering may be good enough.

If you have good aeration in your chemistry laboratory you may want to use chemical mutagen by yourself. In case of chemical mutagen, e.g. Ethyl methanesulfonate (EMS), very high mutant induction rate may be expected. But good care should be taken as most of the chemical mutagens are also known as carcinogenic agents. Please refer to the introduction, or description for maize.

In case of EMS, alkaline degradation of the agent may be suggested. Prepare a plastic bucket in an aerated fume chamber and make NaOH solution of about 0.1% or so. Put all the tissue papers, glass wares, tweezers and waste/rinsing solutions in it for at least one week. Then discard the alkaline solution after appropriate neutralization with acids (HCl etc).

3) How to decide treatment dose

Basically the same way as described in RICE, but instead of seedling height, fresh weight of two week seedlings may be used. For official presentation, use of fresh weight or dry weight will depend on the facility available. For quick assessment, fresh weight will be good enough. Good growth and survival should be guaranteed by small but clear decrease of the growth, by 20 % or less.

In case of chemical mutagen treatment, solution uptake by the seeds may not be much when compared to beans (they imbibe very large amount of water). But the damage by the soaking treatment to the mutagen solution should be carefully examined.

In case of irradiation, dry seeds may be left for a few days before sowing, but wetting of seeds by chemical mutagen treatment may need immediate sowing. There are reports describing about drying back after soaking treatment, but without making preliminary tests under your own conditions, it will be better to sow the wet seeds immediately. Please remember that after pre-treatment soaking and mutagen treatment seeds might have started to germinate.

4) How to grow and harvest the M1 generation

In any mutagenesis work, M1 generation is important only to advance the generation to M2 (or M1V2 in case of vegetatively propagated plant). Therefore, **as far as good amount of seeds can be harvested from M1 plant**, high density planting or any other savings in the field work may be admitted. Usually each M1 plant should be recognized as different individual M_1. Pest control and other good maintenance should be performed to assure good harvest of seeds from the M_1 plants. Market quality of the produce (fruits) is not important.

The population size of M_1 may be more than a few thousands, in most of the cases. Suppose a mutation frequency of 0.1%, treatment of 1000 seeds will produces one mutant or not at all. There was a survey in Japan showing that in most of the successful mutagenic treatment, the M_1 populations were more than 10 thousands. It may sound very large, but it may be achieved if market quality of the harvest is not considered, and high density planting can be practiced.

Depending on the reproductive nature of the plant species, i.e. self-compatible or not, pollination system may differ, but most of tomato and hot peppers are self compatible. If insect shield is available and seed/fruit can be obtained under such condition, it will be the best. Self pollination in M_1 generation is the fundamental factor in seed crops for mutant induction and selection.

M_1 plant may often show somatic mutation sectors in its leaf. Even if the seeds might be complete pure line or in other words, complete homozygotes, it may still show such somatic mutant sectors, albino, yellow or light green color. Suppose a case of somatic mutation frequency of 0.1%, also occurs in the other counterpart of the same locus, as a possible simultaneous mutation. Occurrence might be 0.1 % x 0.1 %, that is one in 1,000,000. This may suggest that in every one million treated cells, the two simultaneous mutation may occur. In case of M_1 plant, it may correspond to one in 100 plants or so. Such mutant sectors in earlier leaves will be wasted before incorporation to M2 seeds, but they may be a very good indication of successful mutagenic treatment.

Although the somatic mutant sectors may be a good indication, but please do not start selection of mutant phenotype in M_1 generation. Most of the induced mutant genes are still hidden in the tissues as they are recessively inherited.

Harvest of the fruit may follow the case described in rice. But perhaps, single round harvesting may be appropriate in most of the cases, as the seed characters are not so important like rice and other cereals. Mutations expressed in the seeds may not be important in these seeds, unless it may be used as a genetic marker. After the harvest of well ripen fruit, seeds should be separated/washed/cleaned and separately stored in small coin envelops for every M_1 plant, until sowing.

5) How to screen the wanted traits

In this crop group, mutant character expressed in the M_2 generation will be important. So, the seeds harvested from M_1 plants should be sown and carefully assessed. Supposing 3:1 segregation ratio, ten to 15 seeds from each M_1 plant may be suggested to find more than two mutants in the segregating M_2 family. In mutation breeding procedures, there is a theory suggesting to sow one grain from one M_1 unit (ear/panicle) for better economy in the field work. But to find the same mutant phenotypes in the same family will give good confidence to the researcher, no doubt.

The sowing and/or transplanting system will depend on the institutional system. But the first mutant recognizable will be albino (white: Chlorophyll-less) seedling. In practical agriculture, it may be of no use at all, but albino seedling will be a very good indication of the successful mutagenesis treatment. At the seedling stage, dwarfness may be also recognizable if so intended. But perhaps in most of the cases, disease resistance and/or fruit quality or fruiting-type/plant-type may be the objectives.

M_2 plants should be planted out to the screening fields as much families as possible, but here again, market quality may not be so important. Suppose a mutation frequency of 0.1%, 1,000 M_1 families might not give promising fruit quality but 1,001th plant might bear interesting fruit. The breeder's eye to examine the plant character will be, of cause, important. Detailed quality examination should be done after market quality cultivation in M_3 or later generations. If you find a promising mutant, then make backcross to the original line to exclude possible accompanied "unfavorable and simultaneous mutant gene(s)".

If you have good screening field, e.g. disease infested field or other abiotic stressed fields, gross survival screening may be performed. Mix your seeds and sow in the screening field, then critically observe and select the survived plant. Often rescue transplanting the survived plants to normal propagation field to harvest the mutant seeds should be considered.

6) How to brush up the mutant

As often mentioned, mutagenesis treatment might induce also other unfavorable mutations simultaneously induced in the same materials. In seed propagated crops, they may be cleaned out by backcross to the original parental line. It requires one more generation to purify the mutant line, but still the effect is clear, no doubt. This kind of cross to the original line is to retrieve the original genotype except for the mutant gene, such may be considered as the case of "direct use of mutant" in breeding project.

Mutagenesis in Tomato and Capsicum

Tomato, capsicum (hot pepper), egg plant are very alike in the mutational work. Only in the capsicum work, you must be very careful about the very hot component contained in the fruit material. Otherwise, genetically the fundamentals are the same as in rice.

This picture of tomato M1 seedlings shows light colored leaf sector in M1 plant. As shown in this picture, all the other seedlings are normal. The occurrence of such mutant sector on M1 plant might be the same mechanism as suggested in other part of the descriptions, e.g. in maize.

This capsicum seedling also shows white or light green mutant sector on the M1 seedling. If only part of the leaf (upper side or lower side) was affected, the color might be looked light green.

The chlorophyll mutant sectors on M1 plant need special genetic explanation but the one shown here might be understood as some kind of physiological mutagen damage, or kind of damage by the agent/drugs (in this case, EMS). Abnormally developed leaves suggest the treatment was effective to give damages, but genetic effect is not suggested.

Mutant searching is again a mass screening job. In this experiment, albino mutant in the same genetic line was sought, and several mutants could be obtained.

Several albino seedling could be detected

Generally, albino seedling may have no economic values, but for academic studies or visual cell markers in case of cell fusion experiments, it may be a good visual marker.

In tomato or *Capsicum* albino mutant grafted on normal green plant can keep growing and give seeds if self pollinated.

5. Cucumber and melons

This group is generally monoecious but male and female flowers are separately developed. In most of the plants, they are self compatible but when the seeds which has developed to have multicellular seed embryo, mutagen treatment of seed will give severe chimerism, i.e. this side is a mutant but the other side is non-mutated normal tissue. To overcome this chimerism problems, two systems may be suggested.

1) Seed treatment.

Seed treatment may be done like other seed crops, rice or barley *etc*., but cut back the vine twice to give tertiary vine. The chimerism might be reduced in the tertiary vine, so that the self pollination within the same tertiary vine may be comparable to true self pollination, perhaps segregating mutant in the next generation (M_2).

2) Pollen treatment to suppress chimerism in the seed embryo.

In order to suppress chimerism in the seed embryo, irradiation of pollen/male-flower was successful (Iida and Amano 1990 Gamma Field Symposia No29 95-111). However, for this method, irradiation facility should be at hand at the time of flowering.

In this method, higher radiation dose may reduce the fertile seed of the fruit. It may be difficult to assay appropriate radiation dose, but theoretically most recommendable method, although radiation source must be at hand.

In the following practical suggestions, method 1) above will be described, as the method may be comparable to other seed crops, except for the chimera ceasing method.

1) How to prepare starting materials

In some crops of this group, hybrid seeds may be soled in market. But as explained in the Tomato Section, inbred line should be selected. If time allows, confirmation of artificial self pollination in an isolation plot is strongly suggested. In market, young fruit of cucumber is sold, but for harvesting of seeds, the fruit will grow much larger. The number of seeds needed in preliminary experiment and for the main experiment(s) should be carefully planned.

In watermelon, seedless fruit may be produced from triploid plant, but it may not be a reasonable material for mutation induction. If a good mutant line could be selected it may be used in such very special purposes.

2) Mutagens suggested

The seeds of this group are usually uniform and flat, except for some pumpkins and squashes. They may fit for ion beam irradiation, but perhaps gamma-rays and X-rays may be suggested as convenient mutagens. In maize, a very strange and special plant, chemical mutagens should be used, but in other materials, like this cucumber group, gamma-rays or X-rays will give good penetration and results.

If chemical mutagen can be used, the water uptake in the early phase of soaking treatment should be carefully assessed, although the water uptake will be not so large as in beans. For the method of water uptake assessment see the RICE Section

3) How to decide treatment dose

The dose assessment idea and method are comparable to the RICE Section. But in stead of seedling height, fresh-weight or dry-weight may be used as described in the TOMATO Section. Generally, 20 % decreased level after appropriate growth of the seedling, may be used as the dose will show clear decrease as the treatment damage but still showing good growth to give necessary M_2 seeds.

The description here are for the method 1) in the introduction. So a good growth to give the tertiary vine with good male and female flowers must be expected. Therefore a little less than 20 % decreased dose may be advised.

In case of chemical mutagens, the same will be applied.

4) How to grow and harvest the M1 generation

As mentioned before, self pollination within the same tertiary vine should be considered. Other cultivation system may follow the description in the TOMATO Section. One practical difficulty here may be the plant shape and the management of how to cut back the hundreds or possibly thousands of M1 plants. The long vine and the heavy fruit for seed harvesting should be considered in the field management.

As described in other Sections, M_1 plants and fruits may not be a market quality, but should be a good M_2 seeds producer. Perhaps one self pollinated fruit from each treated seed will be more than good enough to obtain the M_2 seeds. Seeds should be cleaned separately for each fruit i.e. M_1 plant and separately stored in (small coin) envelope.

5) How to screen the wanted traits

In this group of crops, the target traits may be plant type, fruit quality, disease resistance, abiotic stress resistance *etc*. However, M_2 planting for examination of the traits are basically the same as TOMATO or RICE *etc*. But here the field management/planning is important as it takes much area compared to RICE or BARLEY. But please be noted that the mutant phenotype is expressed in the self pollinated M_2 generation. A possible economic planting will be dense planting only to detect the mutant phenotype, neglecting the market quality.

In case of disease or abiotic stress screening, dense planting and survival selection may be applied by use of pooled M2 seeds.

In either systems, family planting or pooled M_2 seeds, again appearance of albino seedling will be a very good indication of successful mutagenesis treatment.

If you can obtain viable yellow green plants or spine/hair-less mutant which may be used as a genetic marker, it may be useful in demonstrating the chimera tissues after the mutagen treatment. It may be also useful as a marker for a variety newly developed. If not desired in the future breeding procedures, a single backcross may exclude the marker-mutant gene.

6) How to brush up the mutant

If any promising mutant could be obtained, make at least one backcross to the original line to exclude unfavorable simultaneous mutations, as often described in other Sections.

Mutagenesis in cucumber

Cucumber and other melon group has male and female flowers separately like maize. The difference may be the flower bearing position on the plant. When a multi-cellular seed embryo was treated by a mutagen, chimeric mutational sector might be developed on the M1 plant, making self pollination within the plant not a true self pollination in terms of mutagenesis work. To overcome this difficulty, there may be two plans applicable; 1) mutagenesis treatment before seed embryo development, and 2) reduce chimeric tissues by pruning (cutting-back) and pollination within the tertiary vine. Pictures shown here were examples from the former plan. Male flowers were collected in the morning, irradiated by gamma-rays and then pollinated to virgin female flowers. Thus, each seeds developed might be different, in terms of mutational event, but within the seed embryo, all cells might have the same genotype, so that the self pollination within

Figure 1. Albino cotyledons.

Figure 2. Light green cotyledons 1.

Figure 3. Green and wavy streak white cotyledons.

Figure 4. Round heart leaves.

the plant, will be a real self pollination segregating the seedling mutant shown here. Again, albino seedling may be a very good indication of the mutagenesis.

However, for this kind of treatment, the researcher must have irradiator at hand. The second suggestion may be a good possibility for ordinary field breeders, as he/she can ask the treatment to other institution or he/she can treat the seeds by chemical mutagen in their laboratory.

This is a normal leaves of cucumber.

By the Plan 1 method (treatment of half generation upstream) induced leaf shape mutant.

The mutant is the same line as the lower right of the our pictures, Fig.4. The quality of the fruit was the same as the original, but by this mutation, we could get a good marker gene for the leaf shape. The actual effect is unknown yet, but the twines have branches (seen at the lower left corner for example), compared to the original line which has no branched twines.

6. *Chrysanthemum*

Chrysanthemum may be a good model representing vegetatively propagated crops. Ordinary varieties are hexaploid but having good heterozygosities in its genotype, making mutation work rather easier. In vegetatively propagated plants, tissue culture techniques are often very useful methodology. In triploid and seedless banana, in which ordinary propagule unit is very large and difficult or often impossible to irradiate in a gamma-hot cell by large numbers, the late Dr. F. Novak of IAEA (Seibersdorf Lab.) developed adventitious embryony techniques, by which thousands of tiny embryos can be irradiated in a pair of Petri-dish.

Presently, mutagen treatment may be possible in almost all plant species, but in some plants the screening/selection methodologies may still to be developed, or in others still very laborious or need much efforts. Here, Chrysanthemum was taken as a model plant.

1) How to prepare starting materials

There had been many successful reports on inducing flower color/shape changes and plant type changes. However, the change/mutation depend on the genotype, i.e., how much heterozygous, the used line was. The popular cultivars may be hexaploid, a difficult case in mutation breeding, but if your material is *aa*, *bb*, and *Cc*, mutation from *C* to *c* will give *cc* color. But if your material was *aa*, *bb*, and *CC*, one *C* mutated to *c* will still show *C* color, until you mutate both *CC* to *cc*. Therefore, it may be strongly suggested that before starting full sized experiments, you should make preliminary mutagenic experiments with high dosage and examine if your material can give mutant color sectors or not. The color sector or patches may be only a few mm or even microscopic size. If high dosage of the mutagen still did not give favorable color SPOT, it may be better to give up the line and change to other line.

This preliminary examination to see the possibility to change the color of ornamental plants may be common to all the plant materials of this type. For this preliminary test, high mutagen dose may be applied as high as it still give a few flowers, on which small mutant sectors can be examined. In the full scale experiment, the dosage should be kept as small as possible, and as many mutant as possible should be selected for the best performances. Please be noted that in these plant group, exclusion of unfavorable mutant genes induced simultaneously can not be done by back-crossing like the case of rice or other seed crops.

Chrysanthemum is vegetatively propagated plant, so its propagation may need only good labeling work. One cutting of about 10 cm long may have ten or more axillary buds if fully grown. So, for acute irradiation, at least one hundred cuttings may be produced for better results.

2) Mutagens suggested

Many good results have been reported by gamma-rays or by ion-beams. In cases of gamma-rays or X-rays (hard X-rays, more than 150 keV or so) irradiation sample may be bundled, if thickness of the sample is thin enough in terms of distance from the radiation outlet. In case of ion beam radiations, very often penetration and linear energy transfer, and Bragg Peak position should be considered. For these physical factors, the breeder/biologist should consult well to the engineer/physicist.

Compared to these ionizing radiations with high penetrability, chemical mutagens may need good examination of treatment methodology. It may be applied to cultured tissues in the culture bottles, but for that purpose, sterilization of the mutagen solution perhaps through filtration by Millipore system may be required. Most of chemical mutagens may be degraded by autoclaving.

Recent studies suggest tissue culture procedures especially when callus culturing for certain period of time is included, inherent retro-transposon may be activated and produces insertion-mutation in the material. Such retro-transposon induced mutation may be stable in the traditional cultures, or perhaps simple *in vitro* meristem cultures. It may be reactivated only by the next callus induction and culturing.

3) How to decide treatment dose

Chrysanthemum and other vegetatively propagated crops are difficult materials to examine or describe the optimum or favorable mutagen dose. In literatures, there may be various suggestions in measurement of the treatment effect. After the most popular irradiation work, fresh/dry weight may be measured as indication of retarded growth. Again, like in the case of rice (seedling height after some growth period), the dose which retarded the growth to half is too strong for mutant screening. In case of seed crops, back-cross to the original line can exclude unwanted mutant genes, but in vegetatively propagated crops, the procedure can not be applied. Therefore, the selected promising mutant line may carry the simultaneously induced mutant genes.

One most probable way to escape from such drawback may be 1) low dose treatment to reduce accompanying mutant gene, 2) select as many (independent) mutants as possible even with the same phenotype. For example, if a yellow color is wanted from a pink flower line, select two or three or more independent yellow colored mutant line, e.g. yel-1 from cutting No3, yel-2 from cutting No24 and so on. Then take the best one as the best performing mutant line.

As for the gamma-ray dose, experienced nursery breeder once suggested the use of only 250 rad (= 2.5 Gy). The value is much far below the RD-50 (reducing dose for 50%). Such small dose may require large population in M1V1, M1V2 etc. but no doubt the accompanying unfavorable simultaneously induced mutant genes may be reduced very much.

Please be noted that in the preliminary experiments described in 1) above, rather very high dose is suggested to test the line. But when you start your full scale project, use as low dose as possible in your field capacity to incorporate as the screening materials. If low dose is used, normal non-mutant (perhaps 99.9% or more!) plants may still be put to the flower market to save/earn necessary budgets.

4) How to grow and harvest the M1 generation

In vegetatively propagated crops, selection is directly made in the field. In case of pest resistance searching, inoculation or exposure to the pests (disease or insect) may be required, but plant/flower color, shape/type of plant or flower may be selected directly in the growing field. The selected plant may still show chimerism in M1V1 generation field, but perhaps in M1V3 or later population chimerism may not be shown. In most of seed crops, plant generations are renewed rather automatically, but in vegetatively propagated plants, the original M1V1 plants may be still alive in the field. If cut back in rather hard degree, the newly grown shoot may be used as M1V2. Such use of M1 plant depend on the condition/capacity of the field. Anyway, screening of the chimera-free mutant line should be done in M1V3 or later generations.

5) How to screen the wanted traits

In Japan, the black-spot-disease resistant sport of Japanese pear `Nijisseiki` was found on a tree, planted in a gamma-irradiation-field and irradiated by chronic gamma-rays for almost 20 years. However, in this case, the period may be considered as the time necessary to be screened as fully grown mutant sport. Mutation occurs in a single cell, then it grows into larger and detectable size. In vegetatively propagated crops, this kind of time lag to reach selectable size of the tissue is necessary.

In this black-spot-disease resistant sport of Japanese pear, registered and released as `Gold Nijisseiki` was then used to develop toxin-screening-method which accelerated the other two resistant lines in the Japanese pear.

In case of flower colors of *Chrysanthemum*, petal tissue of a new mutant color sector can be cultured *in vitro* to let it be a single re-differentiated plant.

6) How to brush up the mutant

In this vegatatively propagated crop group, vegetative generation advancing from M1V1 to M1V3 or more is inevitable. To shorten the period, tissue culture method as a tool for obtaining chimera-free mutant tissue may be useful.

Even if mutant tissue can be selected in M1V3 or later generation, the structure of a meristem (growth corn) may still suggest some kind of instability which may be observed in the practical agricultural field. To avoid this rather inherent chimerism in the meristem, some people suggest the use of tissue culture to obtain chimera-free mutant line. To obtain chimera-free tissue, callus formation at least once will be the easiest way, but elongation of the callus period may result in activation of retro-transposon as mentioned above. The period of the callus culture should be kept minimum.

Chrysanthemum is preferred flower all over the world.

Colors, shapes, growth habits are all breeding objectives for long time incorporating spontaneous mutations. These are example of large flowers in Japan.

Some cuttings may be irradiated then put to soil to give a clone, then often color mutant sector appears as this picture. However, please be noted that the mutant sector is a somatic mutation sectors and the appearance may strongly depend on the genotype. If completely homozygous and polyploid, mutant sector may not be expressed. To start your work, you should run a preliminary experiment whether your material can give somatic mutant sector or not.

This is a case of white mutant sector developed on a pink flower variety.

Red mutant sector in yellow flower variety.

Left lower portion of the yellow flower variety, red mutant sector/flower is seen.

Yellow mutant sector on white flower. Perhaps this white color might be a suppressed form (dominant) of yellow color, due to a dominant color inhibitor gene, inactivation of which revealed the hidden color of yellow.

An example of yellow flower on white variety. Mutant colored sector may be cultured on appropriate medium and mutant line may be developed.

A private nursery in Japan developed six color variant having the same plant and flower types. They named them "Bio-giku-Rainbow". In the close up picture, lower left is the original variety.
The lower picture shows the view of flowering season. Plant type and flowering times all very alike.

7. Difficult Crops

Genetically and basically there may be two fundamental applications. 1) mutation induction in homozygous crops through Mendelian Segregation of mutant homozygotes; 2) mutation induction in already heterozygous genotype, by inactivation of dominant gene. In rice or in some variety of Chrysanthemum, it may easily be applied, however, very often application seemed very difficult, due to 1) very long life cycle to obtain THE SEGREGATION, 2) unknown genotype; 3) dominant gene/phenotype desired *etc*. In the last case, induced mutation methodology may not be appropriate, and other method e.g. gene transfer by cross-breeding (and repeated back-crossings) or gene (DNA) transfer by transformation method.

In case of **"very long life-cycle"**, tissue culture may be helpful but it may not a decisive method. Special long term project may be needed. For the case of **"unknown genotype"** a preliminary experiment with higher mutagen dosage (LD-50 for example) may be helpful to examine the possibility of occurrence of detectable mutant sector. If you get some positive reaction out of the high dose experiment, then reduce the mutagen dose to ordinary lower level (half or less of the LD50dose).

For self incompatible crops like buckwheat or radishes/*Brassica*(2x) see the next section of "8 Self incompatible". In these crops, Mendelian segregation may be difficult to have. Experiments in *Drosophila* fly or mouse and/or mammals (extreme case of self incompatible organism) may be a good reference.

1) How to prepare starting materials

Preparation of the starting materials may be the same as the other cases. To leave the untreated control will be necessary. Materials should be prepared as much as possible including preliminary experiments. The researcher should be good in handling of the material plant, both in nursing and observations.

2) Mutagens suggested

X- or gamma-ray irradiation will be the easiest mutagen with its high penetrability to the plant materials. These radiation may not leave induced radioactivity like the case of neutron irradiation.

Some chemical mutagens are known to have much higher mutagenic action, but application may need various know-hows in its application *in vivo* or *in vitro*.

3) How to decide treatment dose

In case of the "preliminary experiment" suggested above, high dose of around LD-50 (the dose by which 50 % of the material will be dead after some period) may be suggested. But in the project application, about 20 % down dose, described in "Rice", that is far less than LD50 dose, should be used.

4) How to grow and harvest the M1 generation

This may depend on the reproduction pattern and genetic system of the material. If your material is heterozygous, or positive reaction in the preliminary experiment, you may start selecting in the M_1 generation.

If your material has very long life cycle but self compatible, basically follow the rice plan. In other cases, refer to other plans suggested.

5) How to screen the wanted traits

If you can detect any albino seedling, it may be the first step of the success. Then proceed to screen any disease resistance or abiotic stress resistance. This general strategy may be applicable in most of the cases, however, in rice bacterial blight resistance screening, the powerful chemical mutagen, EMS, produced many albino seedlings, but no resistant seedling was found (Dr. K. Nakai (personal comm.)). With reactor thermal neutrons he obtained many resistant mutant. This may be an exceptional case and need further studies, but one possibility may be that with point mutations (induced by EMS) may still produces some nourishing proteins, but with the high LET radiations, like neutrons, the deletion type mutations may not produce the nourishing proteins

necessary for the pathogenic bacteria.

6) How to brush up the mutant

Simultaneously induced mutation may occur like other plant group. The same methodology may be applied here also. But to overcome the difficulty of the very long lifecycle, try to induce and screen as much mutant as possible, in search of better mutant which may perform well without laborious back cross and other work.

Orchid and other examples

Difficulty in Mutation Work may be due to 1) very long life cycle, 2) self incompatibility, 3) polyploidy and 4) seedlessness, and/or their combinations.

Den. pharaenopsis is a very important exporting flower in the tropical Asia. Variety improvement has been done by cross-breeding incorporating color variants and some remote species. The difficulty mainly is due to the period to reach flowering, but *in vitro* culture system is very well developed for these plants and mutant induction may depend on further endeavor of researcher/breeder.

Most of the ornamental orchids have typical (normal) shape of the species,

but in some oriental orchids, abnormal double flower like this "Yogo-chou" (above picture) is highly evaluated.

Normal shape is like the left one, (This variety lacks dark dots, which is usually seen in the wild type).

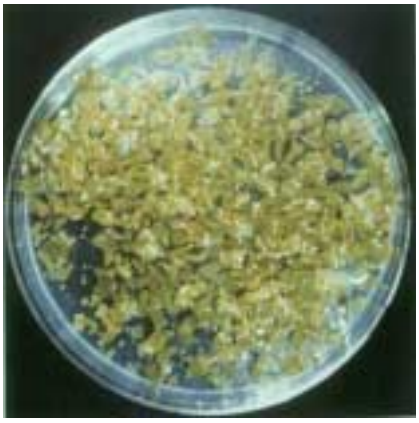
Self-incompatible plants like buckwheat (*Fagopyrum esculentum*) may carry some mutated gene in it, so that after mutagenesis chlorophyll mutant sectors may be seen like this. Usually the border of the mutant sector is very clear and the pattern indicates the developmental pattern of the tissues.

Oilseed rape (*Brassica napus*) is tetraploid and self compatible, but radish and most of other diploid Crucifer vegetables are self incompatible. However, old landraces have been developed perhaps by incorporation of natural/spontaneous mutations. Famous two land-races in Japan are shown here.

Thin but very long Moriguchi-Daikon Late, thick but very large radish two Sakurajima-daikons are seen here (compare the size with the lady's foot).

Sweetpotato is hexaploid and self incompatible. As a mutagenesis trial for this plant, leaf petiole dipped into chemical mutagen solution (EMS) seemed to give small root-bulb which may then planted in the field for searching mutant sport/sectors.

The seedless plant like banana is still possible to improve by mutation, if embryoids can be induced by tissue culture, hundreds of plant can be irradiated in one Petri dish. The techniques had been developed by Dr. F. Novak in IAEA laboratory in Seibersdorf, Austria.



8. Self incompatible crops

Some crop plants have out-crossing nature, which disturbs the usual mutant selection procedure in M_2 generation. Cucumber, melons and maize plants are grouped in this out-crossing/out-pollinating, but they are self-compatible, that is, by artificial pollination, we can expect mutant segregation in the selfed M_2 progenies. However, some plants are SELF-INCOMPATIBLE and need special handling in detection of mutant plant. Diploid *Brassica*, radish (*Raphanus*) and buckwheat (*Fagopirum*) are such difficult plants. At the last part of this section, an example tried to break through this difficulty in feeding crop is attached for your reference.

Self incompatible true out crossing crops will react in the mutagenesis experiments like animals or insects. Therefore it may be a good Idea to use mutation experiments in these, e.g. *Drosophila*. The Idea is to mimic small population cage system. Treated M_1 plants may be allowed to pollinate freely, but in a small population group. The attached reference suggest such small population groupings. By that way, within a subgroup after a few generations, recessive homozygotes will start segregation. This may be observed by production of albino seedlings in the progeny. Albino plants are useless in crop improvement work, but it is a very clear indication of successful mutagenesis treatment done and very appropriate generation to detect the mutant plants.

But, please be noted that the number of subunits in an experiments may amount to more than hundreds. Suppose a mutant frequency of 0.1 % and one subunit may contain ten treated seeds for starting, 100 subgroup might have 1000 treated seeds, then you may find one mutation (of a few plants, perhaps) or not at all.

Another possibility may be to induce self compatible mutation at first, then apply the method used in rice. Theoretically a very good Idea, but at first, you should search a reliable reference.

Buckwheat is well used in Japan making good noodles from the flour of the harvested seeds. Once I tried to induce waxy starch in buckwheat, expecting the mutant character will be expressed in pollen grain, which is haploid, so I examined pollen grains in the buckwheat field. Theoretically it should work well, if other deskwork businesses did not disturbed me. If such mutant could be found, the branch or the plant will be marked and subpopulation will be planted to have segregation in the following or in later generations.

In radish, bud pollination may give selfed progeny, but hundreds, or more, of bud-pollination will be a tough work.

If your target phenotype can be detected in cultured tissues, in vitro culture and direct detection may give some results. But again, the mutant phenotype may be suppressed by the normal non-mutant dominant gene in M_1 generation. Very often the planning on a paper/desk is very useful in the experiments in genetics.

1) How to prepare starting materials

In this group of crops, very often the line purity is questionable. But if the genotype impurity is so large to disturb the mutagenesis experiments, ordinary selection breeding will give good results before mutagenesis work. Please examine the deviation of phenotypes well before starting the mutation induction experiments.

Even if the deviation of the phenotype is not large, perhaps some genes are in heterozygous condition, especially in chlorophyll genes. Then, the mutagen treatment will give chlorophyll deficient mutant sectors in leaves, much frequently than rice or tomato *etc*. Mutant sectors on leaf may eventually lost and may not be transmitted to the next generation. But the color mutant sectors on leaves will be a very good indication of mutagen treatments.

2) Mutagens suggested

The size and shape of the seeds will differ from crop to crop. Small seeds of *Brassica* may be irradiated by ion beams. *Raphanus* seeds are also small but somewhat critical in penetration of low energy ion beams. In this group of crops, so far no special reaction to ionizing radiation, like the case of maize, is not reported. As for the mutagens X- and gamma-rays may be the most appropriate.

Chemical mutagens may be used also. In this case, the water uptake by the seeds may not so significant

like beans, but before starting the experiment, water uptake experiment should be done at least once.

Pollen culture in *Brassica* should be kept as short as possible, if somaclonal variation is not the purpose of the culture work.

3) How to decide treatment dose

The procedure may be described in other sections, e.g. RICE or TOMATO. Weight may be a good component to see the treatment damages after a few weeks growth.

As for the experimental planning, many dose classes with relatively small numbers of seeds will do well. Please be noted that in seeds high in oil contents seemed to be resistant to X- and/or gamma-rays. In some example, up to 700 Gy gamma-ray are reported as appropriate. So please extend your highest dose up to that dose or higher (1000 Gy). As usual, the highest dose should be determined as the high dose where most of the seeds are dead. The sensitivity against ionizing radiation seems to depend on the inherent repair system and not the size of the seeds.

4) How to grow and harvest the M_1 generation

This group needs extra care in the experimental design. That is why this section is described. Basically small population cage work in *Drosophilla* may be a good model to refer. Plants may be grown in the field according to your scheme (attached reference may show one idea). Accuracy in Genetics should be respected than market quality product.

Please do not attempt any selection, until you can observe albino seedling or other true indications of mutant segregation.

5) How to screen the wanted traits

The last sentence of the foregoing section is important to indicate that you may start selection of mutant. You may plant out the sub grouping as families to detect and/or select favorable/promising mutant plant.

Gross screening in biotic/abiotic selection field may be done with pooled seeds for several subgroups.

There will be one very important factors, when you find ONE promising mutant in the field. Please remember that your material is self-incompatible material!! Therefore make pollination to or from the mutant to the original line or brother/sister plants. The mutant phenotype may be hidden in the next generation, but the mutant gene will be maintained in the progeny and segregate a few generations later.

6) How to brush up the mutant

The usual procedure of CLEANING should be done somewhere before the yield test. Preferably, the mutant should be crossed to the original non-mutant line, as described above. In case of brother/sister pollination, one more back crossing will be suggested as a real cleaning work.

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「穂別系統内交雑法」

他殖性植物における突然変異体選抜方式の開発

"Crossing-Within-Spike-Progeny method"

An Effective Method for Selection of Mutants in Cross-fertilizing Plants

種子繁殖性植物における放射線育種の研究はイネ・オオムギ・ダイズなどの自殖性植物に限られ、他殖性植物はほとんど扱われていない。他殖性植物では照射方法、照射集団の増殖法、突然変異体の選抜方式など自殖性作物とは重要な相違点があるにもかかわらず、これまでまったく解決されていない。このことはとくに牧草・飼料作物など他殖性種の多い分野での突然変異育種の発展を阻んでいる。

大部分の人為突然変異は遺伝的優性から劣性の方向に生じる。放任受粉で維持されている他殖性集団では突然変異遺伝子はヘテロ接合体として存在しているので表現型から検出できない。突然変異遺伝子をホモ接合体として分離させるには集団の近交度を高めることが不可欠である。そのための方式として新しく考案した「穂別系統内交雑法」が有効であることが、イタリアンライグラスおよびライムギを用いて証明された。これは照射集団から穂を採り、穂別に次代系統をヒル栽培して開花期に袋かけにより系統間隔離と系統内交雑を同時におこなう方法である。系統間隔離には労力、経費を要するので、隔離前の世代の突然変異遺伝子頻度を少しでも高めておくことが望ましい。また穂内キメラがあると遺伝子頻度が同じでも突然変異体頻度は著しく低くなるのでキメラの解消が必要である。この両点に対して「累代照射」の併用が有効な解決策であることがわかった。他殖性植物の累代照射では自殖性植物でみられる種子不稔の増大も少なかった。イタリアンライグラスではγ線30kRの6回累代照射によって、葉緑素変異の系統当り頻度は70%に達した。コンピューターによる理論計算から、自家不和合性の他殖性種では、隔離世代の各系統は4個体以上、選抜世代の個体数は10個体が最適であることが示された。この累代照射併用の穂別系統内交雑法は風媒性や虫媒性の各種の他殖性植物の突然変異体を得る上で現在最も有効な方法と考えられる。

（鵜飼 保雄）

写真説明：イタリアンライグラスの累代照射に穂別系統内交雑法を適用するための袋かけによる隔離

Isolation by bagging of spike-progenies from recurrently irradiated populations of Italian ryegrass

表1　イタリアングラスの累代処理集団に「穂別系統内交雑法」を適用したときの葉緑素変異の出現頻度

Frequency of chlorophyll mutations in Italian ryegrass populations recurrently treated with gamma-rays or ethyleneimine.

処理区 Treatment		観察 Observed		突然変異 Mutated		突然変異頻度（%） Mutation Frequency per (%)	
		系統数 Progenies	個体数 Plants	系統数 Progenies	個体数 Plants	系統当 Progeny	個体当 Plant
無照射区（control）		100	34,267	15	113	15.0	0.33
γ線（γ－rays）	10 kR $^{6}M_{9}$	96	96,238	38	848	39.6	0.88
	20 kR $^{6}M_{9}$	100	101,879	59	1,543	59.0	1.51
	30 kR $^{6}M_{9}$	102	90,820	72	1,701	70.6	1.87
EI *	0.05% $^{6}M_{9}$	97	63,429	34	622	35.1	0.98
	0.10% $^{6}M_{9}$	100	75,908	56	770	56.0	1.01
	0.15% $^{6}M_{9}$	100	70,048	50	744	50.0	1.06
γ線（γ－rays）	30 kR $^{5}M_{7}$	288	214,300	133	3,595	46.2	1.67
EI	0.15% $^{5}M_{7}$	213	262,000	161	4,792	51.4	1.83
無袋区** γ線	30 kR $^{6}M_{9}$	100	28,000	10	35	10.0	0.12

* Ethyleneimine エチレンイミン

** Without isolation between spike－progenies

An Effective Method for Selection of Mutants in Cross-fertilizing Plants

The use of induced mutations for improvement of seed propagating plants has so far been confined to self-fertilizing species. The selection methods which have been proven effective for obtaining of mutants in self-fertilizer are not valid for cross-fertilizing plants. In an open pollinating plant population mutated genes are involved as heterozygotes which can hardly be discriminated from normal plants due to recessive nature of most of the mutations. Hence, adopting of a method of inbreeding is essential for obtaining mutants as recessive homozygotes from the treated population. A method which the author call Crossing-within-Spike-Progeny (CSP) Method was found to be very effective for screening of mutants in *Secale cereale* and *Lolium multiflorum*. The procedures of the method are composed of (1) taking seeds separately from each spike of the treated population, (2) sowing the seeds in hill plots in the next generation, (3) isolating each hill from others by bagging all the plants of each hill at flowering time, and (4) taking seeds from each hill and sowing them for selection of mutants. To make the frequency of mutated genes in the population before begging higher and to enlarge the mutated sector within spike make the method more effective. Recurrent mutagenic treatments for successive generations answer these two requirements. Unlike in a self-fertilizer, seed sterility did not increase with recurrent treatments. The frequency of progenies with chlorophyll mutants reached to as much as 70% after 6 cycles of 30 kR of gamma-ray exposures when the CSP method was adopted. Thus, the Crossing-Within-Spike Progeny method accompanied by recurrent mutagenic treatments is recommended for getting mutants in cross-fertilizing plants.

(Yasuo Ukai)

Buckwheat (*Fagopyrum esculentum*) is self incompatible plant. The out-crossing nature has served the plant to hold mutant genes in it. So, mutagenesis treatment very often reveals chlorophyll mutant sectors in M1. Such sectors suggest that mutagenesis treatments has been effective. Induction of self compatible mutant may be a good idea, but inbred-weakness (reverse of heterosis) may give a new problems.

9. Beans

This group of crop is genetically the same seed propagated diploid crop like rice. Therefore, basically refer to the description on rice. However, there are two distinct differences in their behavior or handling methodology.

1) When dry seeds are irradiated by X- or gamma-rays, the dose-response curves and suggested dose applied may be comparable to rice. But if you want to apply chemical mutagen as water solution, the water uptake by the bean seeds are very much larger than rice or other cereals, very often reaching twice or three times more than its original weight. The water uptake measurement and "chemical mutagen dose" experiment should be done thoroughly before the main experiments.
2) Another difference may be the number of M_2 seeds obtained from M_1 plant. To have a clear 3(wild):1(mutant) segregation, about 10 to 15 M_2 seeds from possible mutant sector should be harvested. The size of "possible mutant sector" may be observed as mutant-colored tissue on M_1 plant, if appropriate numbers of heterozygote seeds are mutagenized. Without that information, this may be just a guess, but a fruit/capsule developed in a node may be considered as one M_2 seeds family.

2-1) Some crops have long capsule accommodating many seeds in it, like "yard-long-bean" or "azuki bean". In such cases, one fruit/capsule seeds may be considered as a family which may segregate into expected 3:1.

2-2) The other group may have only a few seeds in a fruit/capsule like "soybean" or "peanuts". In case of soybean, fruit/capsule grown on a leaf node may be considered as a "family", although this should be tested as described above. In other cases, like "peanut", the family may be only 1 to 3 seeds etc. In these cases, the fruit/capsule on the same branch may be grouped (only) for convenience, although 1 to 3 segregation may not be expected.

1) How to prepare starting materials

Most of this group of seed propagated crop may be obtained as pure line. But it may be still advised to plant them in isolated plot to have the starting materials, the true pure line seeds. The artificial pollination may be difficult in some of the plant species, e.g. soy-bean, but artificial pollination will be important when a favorable mutant can be obtained, to clean up the mutant line. For that purpose, some hundreds seeds should be kept away for future back-crossing work. The seeds may be stored under low temperature.

The starting materials may be kept dry until mutagen treatment. Whether the seeds are kept in a humidity controlled desiccator or not may be depend on the purpose of the experiment. If it is to see the reproducibility of the irradiation or other mutagen treatment work, all procedures should be well controlled and well described. But if it becomes a routine treatment in your laboratory, perhaps just air dried seeds may be used.

2) Mutagens suggested

Dry seeds may be sent to irradiation facilities elsewhere in the world, but please respect the plant quarantine regulations in the recipient country. With X-ray or gamma-rays, again, secondary electron equibrium may be important as described elsewhere (Tomato section). With X- or gamma-rays, perhaps sample seeds may b e irradiated from outside of the envelope packages. But in ion beam or other delicate irradiation work, where penetration of the radiation is critical, spherical seeds like soybean will needs extra-care in handling. Also large seeds like *Phaseolus*(beans) or *Arachis*(peanuts) may have penetration problems. Perhaps X- or gamma rays will be convenient, if mutant seeking is the purpose.

If you want to treat the material by chemical mutagens, water uptake and the actual mutagen dose absorbed by the seeds must be carefully examined beforehand. The wasting method of the chemical mutagens needs further extra care for researchers and environmental safeties. This is described in MAIZE or other sections.

3) How to decide treatment dose

Treatment dose may be determined as described in the RICE section. To determine the damages by treatment dose, fresh weight or dried weight may be used, instead of seedling height. Measurement may be done after some growth period, e.g. two or three weeks, after the treatment.

If you want to treat the material by chemical mutagens, water uptake and the actual mutagen dose absorbed by the seeds must be carefully examined beforehand. In the water uptake experiment, about ten to 30 dry seeds may be measured every 30min or one hour for the first day, then perhaps up to the next days. If you fined a slowed down in the water uptake, that plateau period may be appropriate for soaking treatment for three to seven hours at 25 degree centigrade or so.

To measure the growth suppression by the mutagen treatment, allow the treated seeds, irradiated or seed soaked in a mutagen solution, to grow for two or three weeks in your nursery bed. In these preliminary experiments fresh weight or dried weight of the seedlings may be measured like the case of Tomato.

When successfully obtained the preliminary experiment results, either with radiation or chemical mutagen, the dosage of the mutagen may be determined as 10 to 20% growth reducing dose range, where growth and production of M_2 seeds may be expected.

4) How to grow and harvest the M1 generation

M_1 plant may be grown, favorably, in an isolated plot. As often suggested, the growth of M_1 is necessary to obtain the M_2 seeds safely. Therefore market quality may not be important, but control the vines so that every M_1 plants can be recognized. In soy bean or some of other crops having simple erect plant types, it may not be so important, but vine type climbers need extra cares in separating each M_1 plant when harvesting the M_2 seeds. For bushy plants, the same recognizing cares will be needed. Ordinary pest control procedures should be applied to have genetically appropriate harvest.

The harvested M_2 seeds (on M_1 plant) may be put in a coin envelopes separately for each M_1 plant, dried then stored until sowing. Please refer to the M2 family situations described in the 2-1) and 2-2) described above.

5) How to screen the wanted traits

The seed coat derived from maternal tissue, but if you can see through the seed coat, the cotyledons may be examined first, like the case of rice, where endosperm characters e.g. waxy starch or not, can be examined. In cases of seed characters, which one is the M_1 and which one is the M_2 characters must be learned beforehand.

The same M_2 planting patterns suggested in the RICE section may be applied to beans also, although M2 family definition might be different or not be determined yet. In some cases for bifotic /abiotic stress resistance mutant searching, pooled seeds may be planted in the screening field to select surviving plants.

Even in such cases of gross survival screening, sub grouping of M_1 plants may be useful. Suppose you have successfully selected two disease resistant mutant from subgroup #12, you can not tell that they might be come from the same origin/mutation or independent origin. However, if the mutant was found in the subgroup #12 and #15 separately, they are clearly independent mutants of different origins.

6) How to brush up the mutant

Here again, as very often described, to clean up unfavorable simultaneous mutant gene, back crossing to the original line is an important and necessary procedure. In some plant species, emasculation and artificial pollination are difficult, but this is a necessary procedure in brushing up the mutant before formal yield testing.

Please name the mutant with appropriate genetic name, and so in the mutant variety name, when it become the time.

Some examples of soybean and sesame mutants

Soy-bean is useful in very wide applications, for protein, oil and starch. In the field, this plant fixes nitrogen from the air as nitrogen sources, having Rhyzomes in the root. The function may be increased by "super nodulation" mutation like this picture (second from left). The mutant still produced nodules even in nitrogen abundant hydro culture (second from right). Agricultural meanings/effects should be investigated further.

Sesame usually has one capsule at each leaf-node. But in Egypt, they induced three capsule for each leaf node. The total harvest might depend on the amount of photosynthesis, but still significant harvest increase was recorded and the mutant was registered as a good variety in Egypt. Three capsules per leaf node may be known to some people, but new induction in good variety may open a new field.

Normal plant

Three capsules per leaf node.

10. Callus culture

Use of tissue/callus culture method may have several faces to support plant breeding work. Embryo Rescue may be the most significant contribution for the crop improvement. In relation to the mutation breeding work, there may be two ways of contributions.

1) One may be to help easier handling of materials in ordinary mutagenesis work. For example a planting material of banana is too large to irradiate hundreds or thousands of them. The late F. Novak of IAEA, Seibersdorf Laboratory developed a method of embryoid irradiation system, by which hundreds of separable embryoids can be irradiated in a Petri-dish. Another useful help from the tissue culture may be solid mutant tissue/plant production from chimeric tissue.

2) The other important factor is mutant production method known as "somaclonal variation". Some part of this phenomenon can be explained by "activation of Retro-Transposon" as reported by H.Hirochika (1995 Gamma-Field Symposia No.34 77-91 and 1999 in Molecular Biology of Rice pp43-58 Ed. K.Shimamoto, Springer-Verlag, Tokyo, Hirochika 1997 Plant molecular Biology 35: 231-240), and an example had been reported in a IAEA Seminar held in the Philippines in 1999 (Amano et al. 1999 FAO/IAEA Seminar IAEA-SR-210/22: pp27-30).

The latter one occurs when callus culture is extended for a certain period of time, e.g. for a few months. During such heavy stresses, otherwise inactive/sleeping DNA sequence start producing messenger RNA, then the product seems to be modified to change into DNA by the help of retro-replication system producing DNA type transposon. If the callus period was elongated too long, then too many transposon DNA are spreading in the genotype. The reason of albino regenerated plants, often found from prolonged callus, may be explained by this mechanism.

Fortunately, such retro-transposon induced mutant seems to be very stable in ordinary agricultural conditions, until callus induction done again for any reasons. If you do not want to have such somaclonal-variation/mutation, the period of callus state should be kept as short as possible. However, the reported example showed several cases of "all mutant families" in some phenotypes. As mentioned in the introduction section of this Addendum, details are still to be studied. According to the example cases, such non-segregating cases seems to be limited to only a few phenotypes. An explanation by methylation of the gene seems to be very likely, although detailed studies are still needed.

In this Section, Callus culture as mutagenesis method will be described. Embryoid culture and other helping methodology should be referred tissue culture text books/manuals/recipes.

1) How to prepare starting materials

The preparation of the starting materials is the same way as other mutagenesis work, as far as callus induction and plant regeneration system is reproducibly established. If your material, particular variety of a crop, is not sure of the procedures, try to induce a mutant in easier variety, from which you can introduce by pollination, cell fusion or other methodology.

2) Mutagens suggested

In this section, the mutagen is comparable to a stress given to the plant materials, e.g. callus induction and some period of callus culture. Callus induction agent may be of any kind, 2,4-D etc.

3) How to decide treatment dose

In this method, culturing period as callus may be comparable to the dosage/strength. If You maintain only a short period of time, the transposable DNA may not be many, so that the mutant induced will be very few.

If the culturing period was too long, too many transposable DNA might be produced. If you can have DNA analysis Laboratory and have a good DNA marker, then you can try direct analysis of the transposable DNA in your material.

4) How to grow and harvest the M_1 generation

If callus culture period is appropriately short and the numbers of transposable DNA is not many, cells of the regenerated plant have only a few heterozygous recessive insertion mutant genes in it, comparable to the irradiated M_1 plants. The method of detecting and selecting mutant plants is basically the same way as in irradiation method. But please be noted that the generation naming is different in the regenerated plants. The regenerated plant from a callus is in R_0 generation, like the case of transgenic plant in T_0. The insertion mutant genes are "inactivated state", therefore to have a homozygous recessive mutant, one more planting in the field will be needed for the plant type mutation.

If you want seed character mutant, e.g. waxy starch in rice grain, the first segregating generation will be the R_1 generation which is comparable to M_2 seeds in irradiation and other mutagenesis treatment. The naming of the generation may be confusing, but please consider the expected genotypes.

The field maintenance, harvesting of R_0 (=M_1), and planting of the segregating generation of R_1 (=M_2) are the same as ordinary mutagenesis work. Only the generation call is different. Please be careful in reporting your results.

5) How to screen the wanted traits

If you are thin king of the callus-mutation/retro-transposon-mutation as a method of mutagenesis, the detection/selection methodology will be the same as ordinary mutagenesis work, family wise detection or gross pooled planting.

6) How to brush up the mutant

In this methodology, the resulting mutant may be of insertion mutant, which may be searched by the inserted DNA sequence among the all genes in the genome(s). This may be a very precious mutant gene, easily detected by DNA analysis and identified by its phenotype. The mutant plant will be also used as very stable mutant for plant breeding.

Please be noted that ordinary transposon induced mutant is very often unstable and return to the original non-mutant state at relatively high rate. Also many cases of color spots on the flower petals might be due to ordinary transposons. For the academic research work, if the gene can be identified by the inserted transposon, it has enormous value. For ornamental flowers, it may be also meaningful. However, for cereals and other practical agricultural crops, such instability may not be appropriate in the breeding work.

The cleaning of unfavorable simultaneously induced mutant gene should be done as usual. Otherwise, you can not tell which mutant gene was really effective in the later procedures, breeding or academic work.

Callus activation of retro-transposon Callus/tissue culture often brings aberrant plants when regenerated. Hirochika reported possible participation of "Retro-Transposon" for such mutagenesis. By stresses of callus culture, retro-transposon is copied to m-RNA, then reversely synthesized to a piece of DNA which will be inserted to other locus. The inserted locus is inactivated like mutations (gene splitting). This new mutation will be stable as far as ordinary life cycle is maintained.

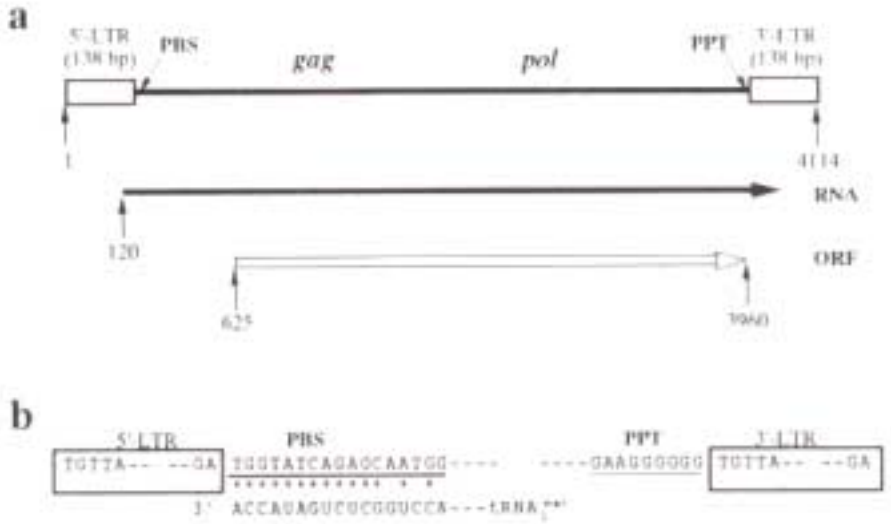


Fig. 1a,b. Structure and expression of *Tos17*. **a** Structure deduced from the complete nucleotide sequence and transcription of *Tos17*. **b** Nucleotide sequences flanking the LTRs of *Tos17*. LTRs are *boxed*, the primer binding site (PBS) and polypurine tract (PPT) are indicated by *thick and thin underlining*. The complementarity between the PBS and the 3'-end of the initiator methionine tRNA is shown by *asterisks*. The *thick arrow* indicates the transcript of *Tos17*, and the *open arrow* indicates the longest open reading frame (ORF) starting with the ATG

Compared to other transposons, which moves around but the number remained unchanged, the retro-transposon increases when the callus stage is prolonged, but the original pieces in the chromosome remains at the original position. In the following electrophoreogramme, the original position shown at the leftmost lane (Control) is maintained regardless of the culture period.

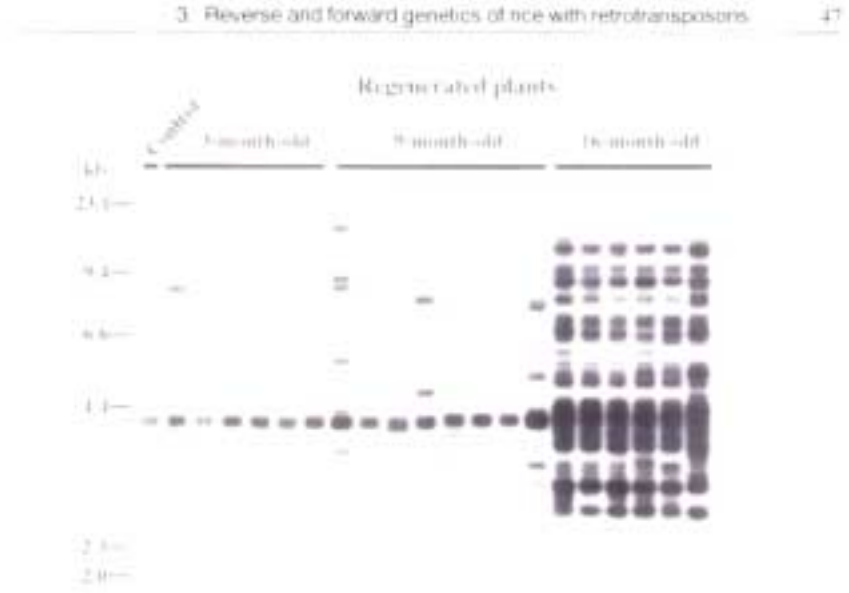
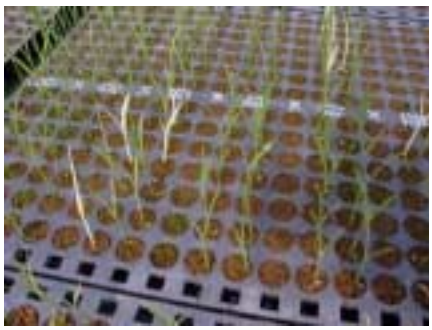
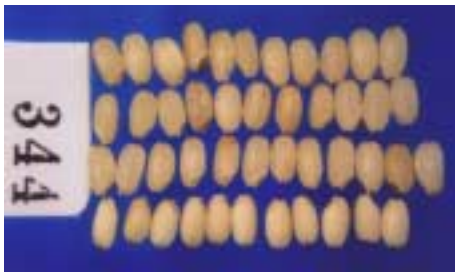


Fig. 2. Activation of *Tos17* by tissue culture. DNAs were prepared from leaves of normally propagated rice plants (*Control*) and plants regenerated from 3-, 9-, and 16-month-old calluses and analyzed by DNA blot hybridization with a *Tos17*-specific probe after digestion with *Mse*I



Albino seedlings were observed as good index of the retro-transposon mutagenesis.

Two stable early flowering mutants are segregated in this M2 field, where M1 panicle families were planted as M2 lines.



Other example of retro-transposon induced waxy starch mutants segregated in 3:1(34:11). In this material, japonica rice, some intermediate phenotypes are seen, e.g. top-row 2nd and 3rd grains, but this is ordinary segregation pattern for them.

There are some strange results like this left marking pole, which indicates all family members shown rolled leaf phenotype in M2. If it were by a dominant mutation, normal plants should be segregated about 25% (or 1:3). Methylation of the locus is suggested, but this should be studied further.

This mutagenesis system is very useful to put a marking tag to the mutant gene. The diseased mutant in this picture (shown by the marking pole) may not be good for breeding purposes, but it may be an excellent material for academic research work on disease infection, as the gene can be isolated by the retro-transposon tag.

11. Fruit Trees

Definition of Fruit Trees may be a little difficult. Here, a wide range of arboreal crops (including Palms) and Banana (botanically herbaceous crop) should be included. But then we will hit many problems. For each target species, the objectives and problems will be different. The only common factors may be that each genotype is very characteristic to each cultivar, making cross breeding very difficult and laborious work. However, in such materials the magic of Mutation Breeding will be very effective in one point improvement （Sanada and Amano 1998）. Another common factor will be the fact, that most of the recessive characters can be modified by mutation PROVIDED if the dominant unfavorable character is in heterozygous condition.

(1) Heterozygocity of unfavorable characterIf the genotype is not known, try with higher doses of mutagens, if it gives any small mutant sectors or not. If there are some possibility of the improvement, then apply mutagen for appropriately lower dose.

(2) Ploidy of the plant/species Many Bananas are triploid, making mutation breeding very difficult, by having three copies of genomes. However, some mutations have been reported, together with some successful mutant cultivars e.g. Novaria and Parfitt (from disease resistant but dwarf Parfitt to normal-sized and disease resistant Parfitt in Australia)

(3) Purpose of mutation work Disease resistance may be wanted in most of the cases, like the successful cases of Japanese pear mutant varieties. However, most of the disease resistant genes are known as dominant genes, where mutation work may not be so effective. The preliminary tests by high dose mutagen described above and quick inoculation tests may be needed e.g. by use of toxin secreted from the disease causing organisms.

(4) Widening of genetic resources In some Seminar Meetings, widening of genetic lines in Mangosteen which has only a few different cultivars/land-laces are reported, were suggested. Parthenocarpy or other vegetative propagation system has disturbed the widening. However, this rather closed vegetative propagation system may maintain spontaneous mutant genes in it. So, mutation work may give some divergence of the phenotype if heterozygous dominant gene has mutated to recessive form.

(5) Seedlessness in Oranges *etc*. Seedless crops might be produced by mutation or by disturbance of meiotic cell divisions. Triploid clone produced by pollination of diploid and artificially produced tetraploid may often be successful in reducing the seeds, like the case of seedless watermelon. Disturbance of meiotic division may be also caused by translocation of chromosomes. This may caused by irradiation of the materials. Homozygote may produce seeds normally, but heterozygotes may reduce their seeds. Seedless grape are produced in Japan by treating the inflorescence by plant hormone solution, but if mutation can bring the seedlessness to the crop, solution treatment may not be needed any more. In the pomegranate, it is said that softening mutation of the hard seed made the fruit easier to eat.

(6) Cross breeding procedure Palm trees are very difficult crop to improve. But if a very long term project is available, irradiation of pollen and ordinary M_2 selection may be done, although not only the time but also the very wide screening field for M_2 may be needed. Tissue culture may not be so useful in reducing the time and the field area, in the experiments.

Basically most of the elements has been described in the other vegetatively propagated crop, for example : *Chrysanthemum*. Perhaps the difference may reside in the major interest, that in the fruit tree improvement, most of the cases are disease resistance, while in the Chrysanthemums color and flower form may be very much interested. Otherwise, the genetic handling of the materials is comparable even though one is arboreal and the other is herbal.

In some fruit trees, the major crop improvement method of cross breeding has difficulties like parthenogenesis, as seen in some oranges, where the seeds are not really the hybrid between the parents. In such cases, reproductive system itself may be the target of improvement. In other cases, like in Mangosteen, widening of genetic variability for any variety development may be the targets.

1) How to prepare starting materials

Refer to the *Chrysanthemum* Section. The genotype of the material is also very important. The successful case of Black-Spot disease resistance in Japanese pear was the facts that their genotype was known already, i.e. the susceptible gene is dominant and the 'Nijisseiki' variety was heterozygous for the gene. Thus, destruction of the single susceptible gene made the disease resistant twig on the tree. The mutant twig was found after 19 years of irradiation in a gamma-field in Ohmiya-town in Japan. Perhaps most of the period of 19 years might be needed to develop the mutant cell growing to the detectable size. Two other Japanese pear mutant varieties could be obtained after a few days long irradiation and toxin screening work.

2) Mutagens suggested

Please refer to *Chrysanthemum* section. If you have large irradiation facility like gamma-field, you may examine the planted materials from time to time. However in most of the cases, irradiation is practically acute irradiation. It may be still useful in inactivation of dominant-susceptible, or dominant disturbing gene to resistant gene actions. In your early phase of experiments, please examine your material to examine that if it is reasonable material for mutagenesis work or not. The idea of genotype appropriate for the mutagenesis work or not has been described in the *Chrysanthemum* section. Use of X- or gamma-rays will be much easier than chemical mutagens.

3) How to decide treatment dose

See the *Chrysanthemum* section.

4) How to grow and harvest the M_1 generation

please be noted that here the promising mutant can be detected in the M_1 generation, but to have a good chimera free mutant clone, M_1V_3 or later vegetative generation, i.e. M_1V_4, or M_1V_5 clones should be tested. Otherwise, market quality product may not be expected in the M_1 field.

5) How to screen the wanted traits

As mentioned above, if disease resistance is your target, inoculation field or toxin screening will be effective. If disease infested field is not so severe to endanger your materials to survive, close observation of the disease symptoms will be needed. Toxin for mutant screening may not need to be purified, if it gives good disease symptoms. Even a crude culturing liquid on the leaf discs may be good enough.

6) How to brush up the mutant

Please be noted that these materials do not fit for the back-crossing to exclude the unfavorable and simultaneously induced genes. In case of 'Nijisseiki' variety of Japanese pear, seed propagation will not give good results, at all.

Perhaps the only way will be that select as much as possible mutant of the same type and select the best one out of them.

Another way of brushing up will be the production of a solid mutant clone out of chimeric mutant tissue. This may be done by tissue culture method. But please be careful the mutant character will not be degraded further by prolonged callus culturing (somaclonal variations may degrade the mutant clone).

Induced mutation in fruit tree

Fruit tree improvement through mutation techniques might be comparable to the case of *Chrysanthemum* flower. If the material is heterozygous, mutant of the gene may be relatively easy to obtain, but if the material is homozygous, recessive mutant phenotype may not easily obtained. In case of Japanese pear, dominant disease susceptible gene in heterozygous condition had been known to the researchers. The researchers developed further the screening methodologies by use of toxin inoculation.

The final cultivar 'Gold Nijisseiki'.

In the case of apple fruit-skin color, perhaps the heterozygous condition of the skin-color was just waited for the mutagenesis treatment.
Comparable results have been obtained in Austria (Europe), too.

For Banana, see 7) Difficult Crops.

Picture files

12. World facilities

……Brookhaven National Laboratory located in the Long Island, New York, USA had open field for Gamma-rays irradiation while plants grown under natural conditions, the Gamma Field. But the design of the Gamma Field had no upper shielding and horizontal shielding bank. The facility had been closed in 1968.

X-ray is also used widely for short period irradiation. Deep therapy type facilities having good power of 250 kV 20mA or above are used often. However, small facilities, as large as the refrigerator, are in the market (mostly soft X-ray type). As far as it has high penetration and can deliver good dosages, they may be used for mutagenesis work.

Small accelerator type 14MeV fast neutron generator had been used in some experiment. Please make attention for the induced radio-activities when you use any kinds of neutrons. In some countries, restriction by the law may be very strict.

IAEA has fast neutron irradiation service, using SNIF (special neutron irradiation facility) mounted in the research reactor of the collaborating Austrian Research Institution. In this picture, beside of the reactor core, an orifice of the SNIF is seen at the lower center can be seen. Irradiation may be done when the reactor is in operation.

Currently operating largest Gamma Field is in Japan. Complete Circular Field is surrounded by 10 m high shielding bank. In this picture, 12 o'clock position, and 57 m from the radiation source at the center produced the black spot disease resistant Japanese pear, registered as 'Gold Nijisseiki'. Toward the right, 1-o'clock are forest trees, like pine or *Criptomeria japonica*, 5 o'clock mulberry, 6 o'clock tea, 8 o'clock, apple tree, 11 o'clock peachs. The yellow patch near the center is a small rice field, which is relatively radio resistant.
The Gamma Field in Japan, still actively used as of 2004.

The irradiation Tower at the center is shown here. The radiation source container is mounted in the air, so that the container itself functions as upper shield. Horizontal beam can be adjusted by a heavy ring filter. The source is remotely operated from the white room on the bank in the background. During the morning, the radiation source is stored in the container and workers/researchers can enter the inside the Gamma Field.

Gamma Field or Gamma Green House is very convenient facility while plant can be grown under the natural condition. However, in the congested area, so called Sky Shine, secondary or tertiary scattered radiation may raise the environmental radiation revel. So, the recently developed chronic irradiation facility in the Kasetsart University in Bangkok, Thailand, utilizes tissue culture technique, which may not need strong illumination on the material plants. The radiation sources are stored within the heavy wall and sent to the irradiation position through the stainless steel guide pipe.

Gamma Room of Kasetsart University, a case of Gamma Greenhouse simulator using tissue culture.

In this picture, some potted plants are seen. As far as the period is less than the start of etiolating potted plant may be irradiated here. Some culture vessels are seen in the right side shelf.

13. Plant for fundamental research

Arabidopsis thaliana has been widely used in genetics experiments. It can be cultured *in vitro* and harvest necessary number of seeds within a test tube.

The culture is easy on agar medium and the culture condition is genetically in complete isolation like *Drosophila* experiments. The mutagenesis work may be almost the same as in rice, in terms of dose of mutagen and segregation in M2 generation. Here, only a few examples are shown.

Ordinary plant type.

Yellow green leaf mutant

Nicknamed as spinach leaved.

Albino seedlings may be the first one you may notice in M2 generation after mutagenesis.

14. Use of chemical mutagen, EMS.

Ethyl methanesulfonate (EMS) is a convenient mutagen to use and relatively easy to handle. However, like all the other chemical mutagens, post-treatment handlings of various wastes should be carefully done as they may be carcinogenic.

1) Storage

Refrigeration is advised by the manufacturer rather than freezing. When taken out from refrigeration, dew drops from the air may damage the chemical. So, use good amount of Silica Gels in a bottle to accomodate the EMS vial. About one or two hours before the use, take-out the entire bottle like this picture from refrigeration and make balance with the room temperature before opening of the EMS vial.

In this picture, a pipetter (Pipette-man) is connected to a glass pipette. A Hypodermic Injection Cylinder may be used in the same purpose. Never try to suck the pipette by mouth for any chemical mutagens.

A water-bath and a shaker will be suggested for uniform treatment. A simple rid/covering with aluminum foil to cover the treatment flask and entire apparatus should be placed in a chemical food to assure safety in the laboratory atmosphere. The left side plastic bucket contains several litters of about 0.1% (NaOH) alkaline water to hydrolyze the EMS waste. Pipettes, flasks, paper-towels, aluminum foils etc. are all dipped into this bucket for at least a week, then neutralize and discard.

General reference Books

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0Introduction

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11 FruitTrees

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